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14. ABSTRACT

Breast: cancer is a genetically heterogeneous disease, and multiple genes remain to be identified among BRCAl and BRCA2 mutation-negative breast cancer-prone families. We believe that a valid approach to identify genetic factors that contribute to breast cancer risk is to evaluate genes coding for proteins that interact with BRCAl in a multiple protein complex. We have recently found one such candidate, referred to as BRCC36. We have reported a profound increase in BRCC36 expression in breast tumors. Furthermore, our studies have defined BRCC36 as a direct regulator of BRCA1 activation and nuclear foci formation in response to IR in a number of breast cancer cell lines. Our results have found that down- regulation of BRCC36 expression impairs homologous recombination repair (HRR) . Therefore, our data suggest that DNA repair pathway activated in response to IR and appears to sensitize breast cancer cells to IR-induced apoptosis. Imgortantly, we found that BRCC36 mutated in the germline of a cancer- prone family and may increase the risk of developing breast cancer. Overall, aberrant expression or mutation of BRCC36 genes in breast tumors may lead to disruption of the normal function of BRCAl and contribute to the development of breast cancer.

15. SUBJECT TERMS
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INTRODUCTION

Extensive mutation testing has indicated that not all "high-risk" breast cancer families are due to mutations in *BRCA1* or *BRCA2* (Bove, et al., 2002; Ford, et al., 1998). Linkage analyses have been proved unsuccessful to identify other breast cancer susceptibility genes (Smith, et al., 2006). The failure of linkage studies suggest that genes with penetrances similar to *BRCA1* and *BRCA2* are unlikely to exist, or at least that pathogenesis mutations in such genes will be very rare. A more plausible hypothesis is that residual genetic susceptibility is driven by variants at many loci, each conferring a moderate risk of breast cancer (Antoniou and Easton, 2006). There is mounting evidence to support the speculation that there are additional, less prevalent breast cancer susceptibility genes (Antoniou, et al., 2001; Kainu, et al., 2000; Seitz, et al., 1997; Thompson, et al., 2002). Germline mutations of *PTEN*, *LKB1*, *ATM*, *TP53*, *MSH2*/*MLH1*, *CHEK2*, and *BACH1 (BRIP1)* are associated with breast cancer, but to a much more limited extent than *BRCA1* and *BRCA2* (Bell, et al., 1999; Cantor, et al., 2001; Chenevix-Trench, et al., 2002; Meijers-Heijboer, et al., 2002; Seal, et al., 2006; Thompson, et al., 2005; Thompson, et al., 2002). Therefore, compelling evidence suggests that other familial breast cancer gene exist, and new approaches must be used to identify new genes that predispose women (possibly men) to breast cancer.

A valid alternative approach is first to examine candidate genes in families with multiple cases of breast cancer, and next to determine whether such candidates carry mutations that might account for a proportion of these families with unknown genetic etiology. The most likely strategy is to evaluate genes that code for proteins with equivalent or complementary functions or function in the same pathway as BRCA1 and BRCA2, such as *CHEK2*, *ATM*, *BRIP1*. Evidence is accumulating that dysfunction of other genes, coding for proteins in the same pathway as BRCA1 and BRCA2, might be important in the pathogenesis of a significant proportion of sporadic, non-familial breast cancer (Hughes-Davies, et al., 2003; Jazaeri, et al., 2002; Turner, et al., 2004). Therefore, this strategy may help to uncover important new insight into the role of BRCA1 and BRCA2 in sporadic breast and ovarian cancer, since *BRCA1* and *BRCA2* are rarely found mutated in these cancers (Futreal, et al., 1994; Lancaster, et al., 1996; Merajver, et al., 1995). One of the major problems of this strategy is that the complete function(s) of BRCA1 and BRCA2 have not been completely established, nor have the majority of the proteins, which interact with BRCA1 and BRCA2, been identified.

Much of the current scientific effort involving BRCA1 is being directed to defining the biochemical functions of BRCA1 and its protein interactions. Wang *et al* have previously reported that a set of proteins associate with BRCA1 to form a large mega-Dalton protein complex, referred to as BASC (BRCA1-Associated Genome Surveillance Complex) (Wang, et al., 2000). This complex includes several tumor suppressors, the DNA damage repair proteins MSH2, MSH6, MLH1, ATM, BLM, the RAD50–MRE11–NBS1 protein complex, and is responsive to double stranded breaks (Wang, et al., 2000). However, it becomes clear that BRCA1 and/or BRCA2 can exist in a number of protein complexes and that several BRCA1/2 associated proteins remain to be identified. Using a combination of affinity purification of anti-FLAG and mass spectrometric sequencing, we have reported a novel multiprotein complex, termed BRCC (BRCA1/2 Containing Complex), which contains seven polypeptides including BRCA1, BRCA2, BARD1 and RAD51 (Dong, et al., 2003). We first reported that BRCC was

an ubiquitin E3 ligase complex exhibiting activities in the E2-dependent ubiquitination of the tumor suppressor p53. In this multiprotein complex, three proteins, referred to as BRCC36, BRCC45, and BRCC120 have been found to be associated with BRCA1 and BRCA2. Reconstitution of a recombinant four-subunit BRCC complex containing BRCA1/BARD1/BRCC45/BRCC36 revealed an enhanced E3 ligase activity compared to that of BRCA1/BARD1 heterodimer (Dong, et al., 2003).

Mass spectrometric sequence analysis identified one proteins referred to as BRCC36. The *BRCC36* gene is located at the Xq28 locus, a chromosomal break point in patients with prolymphocytic T-cell leukemia (T-PLL) (Fisch, et al., 1993). The chromosomal break occurred in two different introns of *BCC36/c6.1A* and the fusion transcripts were expressed at high levels in the leukaemic cells from T-PLL patients (Fisch, et al., 1993). In addition, BRCC36 displays sequence homology with the human Poh1/Pad1 subunit of the 26S proteasome, and with subunit 5 (Jab1) of the COP9 signalosome (Dong, et al., 2003). Cancer-causing truncations of BRCA1 have been found to abrogate the association of BRCC36 with BRCC (Dong, et al., 2003). We have reported a profound increase in *BRCC36* expression in breast tumors (Dong, et al., 2003).

BODY

Task 2: To determine the frequency of BRCC36 germline mutation in BRCA1 and BRCA2 mutation negative breast cancer prone kindreds.

Through the Family Risk Assessment Program(under the direction of Dr. Mary Daly), we have created an extensive specimen bank, housing over 4,000 research participant samples from members of approximately 5,500 high-risk cancer kindreds. Many of these families have participated in studies that allowed for genetic testing of cancer susceptibility genes, including *BRCC36*. To exam *BRCC36* gene for germline mutations, I selected breast cancer-prone kindreds that have tested negative for *BRCA1* and *BRCA2* germline mutations. A set of 11 oligonucleotide primer pairs were designed to amplify genomic DNA region for the *BRCC36*

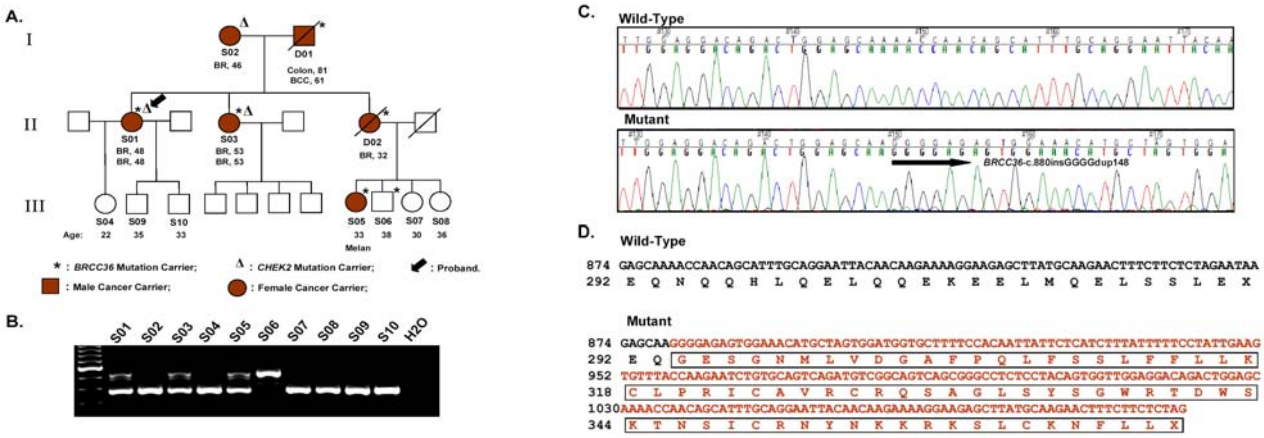

Figure 1. A *BRCC36* Frameshift Mutation *(*c.880insGGGdup148). (**A**) Pedigree of a breast cancer prone kindred with a *BRCC36* frameshift mutation (c.880insGGGdup148). (**B**) Agarose gel image of PCR products of exon 11 for DNAs isolated from the peripheral blood of affected and unaffected family members. Samples from left to right are DNA ladder, PCR products from individuals S01 to S10, and a negative control (H_2O). (**C**) Sequencing analysis for S02 (wild type) and S06 (homozygote *BRCC36* c.880insGGGGdup148 mutation carrier. (**D**) Protein sequence analysis of *BRCC36* c. 880insGGGGdup148 mutation. This frameshift mutation is predicted to result in expression of a mutant protein [i.e., 72 new residues beginning at 294 and a stop codon at residue 366 (p.Arg294ThrfsX73)]. The length of BRCC36 wild type and mutant protein is 316 amino acids (NP_077308.1.) and 365 amino acids, respectively.

coding exons. Blood DNAs from probands affected with breast cancer and reporting at least 2 first-degree and/or second-degree relatives with breast cancer were evaluated for germline mutations. In the initial screen, a frameshift *BRCC36* mutation (c.880insGGGGdup148) was identified in the germline of a *BRCA1/2* mutation-negative breast cancer prone kindred when evaluating exon 11 (**Figure 1)**. The proband (S01) was diagnosed with bilateral breast cancer at age 48 (designated by the arrow). I was not able to test her father (D01, BCC at 61 and colon cancer at 81) and one of her sisters (D02, early-onset breast cancer at age 32) since they died before specimens could be obtained. However, both of these individuals are the obligate carriers because proband's mother (S02) did not carry this mutation and *BRCC36* is located on chromosome X. The proband's another sister (S03, bilateral breast cancer at age 53) also carried this mutation. In addition, the proband reported a niece (S05) with early-onset melanoma, and her DNA sample was positive for carrying this *BRCC36* mutation. Interestingly, we also identified a *CHEK2* c.1100delC mutation in proband (S01), her mother (S02), and her sister (S03). Inactivating mutations in low-penetrant breast cancer susceptibility alleles, such as *CHEK2* and *ATM*, are associated with modest risks (approximately twofold) as compared to *BRCA1* or *BRCA2* mutations (10 to 20-fold by age 60). Therefore, these results suggest that *BRCC36* and *CHEK2* may be cooperating to confer susceptibility to breast cancer in compound heterozgotes.

To determine if a *BRCC36* frameshift mutation results in a stabilized mutant transcript, we have established lymphoblastoid cell lines (LCLs) by immortalizing lymphocytes collected from the proband (S01-mutation carrier), her mother (S02-wild type), and her niece (S05-mutation carrier). The protocol for immortalizing lymphocytes has been described previously (Chen et al, Human Mutation, 2006, in appendix). Genomic DNA analysis by PCR confirms that LCLs S01 and S05 carry this mutation, while the S02 line is wild type (**Figure 2.**).

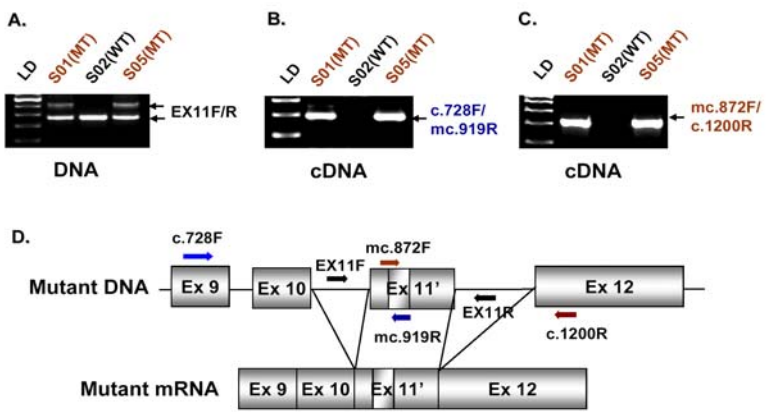

Figure 2. (**A**) Agarose gel image of PCR products of *BRCC36* exon 11 from genomic DNAs isolated from immortalized LCLs. Samples from left to right are DNA ladder, PCR products from LCLs, S01 (mutant), S02 (wild type), and S05 (mutant). Agarose gel image of RT-PCR products with mutation specific primers, mc.919R (**B**) and mc. 872F (**C**). (**D**) Schematics for the splicing of the *BRCC36*-c.880insGGGGdup148 allele.

Total cellular RNAs were then isolated to perform RT-PCR with primers specifically targeting *BRCC36*-c.880insGGGGdup148 mutant transcripts. As shown in **Figure 2B** and 2C, the *BRCC36*-c.880insGGGGdup148 allele is expressed clearly in S01 and S05, but not in S02 LCLs. Following sequencing analysis, a mutant exon 11 (Ex 11'), with an additional 152 nucleotides incorporated, was identified (**Figure 2D**). Because this frameshift mutation affects the sequence of the C-terminal last 23 amino acids of the BRCC36 protein, the commercially available BRCC36 antibody, which recognizes a peptide sequence corresponding to the C-terminal last 20 amino acids, cannot be used. Thus, I am currently deriving a series of new antibodies that specifically recognize N-terminal sequences of BRCC36.

Task 3: To determine the role of BRCC36 in tumorigenesis by studying if its over-expression contributes to malignant transformation of mammary epithelial cells.

(1). Silencing of BRCC36 decreases the expression of MRE11, a subunit of the R/M/N complex

The BRCA1-associated RAD50/MRE11/NBS1 (R/M/N) complex has recently been demonstrated to activate CHEK2 downstream from ATM in response to replication-mediated DNA double strand breaks (Takemura et al, JBC, 2006). To study the mechanisms of BRCC36 involvement in BRCA1 activation in response to the DNA damage, I have first examined whether abrogation of BRCC36 affects the expression of the MRE11 subunit. As described previously (Chen, Cancer Research, 2006, in appendix), HeLa cells were exposed to the siRNA-control or siRNA-BRCC36 twice at 0h and at 48h. Twenty-four hours after the second siRNA transfection, cells were harvested and lysed for Western blot analysis. Results clearly show that siRNA-BRCC36 treatment knocks down the expression of BRCC36 proteins (**Figure 3**). Importantly, although not affecting the levels of CHEK2, depletion of BRCC36 by siRNA targeting significantly decreases the level of MRE11 compared to cells transfected with siRNA control (**Figure 3**).

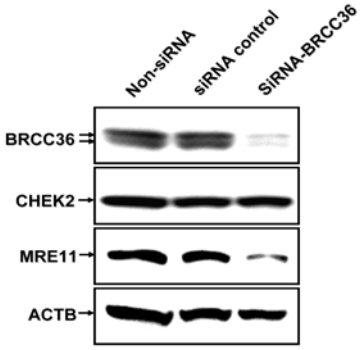

Figure 3. Silencing of BRCC36 down-regulates the expression of MRE11. HeLa cells were mock treated (non-siRNA) or were transfected with siRNA-control or siRNA-BRCC36. The expression of BRCC36, CHEK2, and MRE11 were determined by immunoblotting with specific antibodies. Protein loading levels were evaluated with anti-β-actin antibody.

(2) Depletion of *BRCC36* Impairs Homologous Recombination Repair (HRR)

We have established a functional GFP-based assay to assess specific double-strand break repair. This assay is based on a reporter construct assay [modified from (Wang, et al., 2004)], where a plasmid containing a single 18bp I-SceI restriction site within an inactive green fluorescence protein (GFP) expression cassette is specifically cleaved allowing the detection of homologous recombination repair (HRR) by GFP expression. The pDR-GRP was kindly provided by Dr. M. Jasin, Memorial Sloan Kettering (Pierce, et al., 1999). In addition, since the earlier submission we have generated a lentivirus construct (Lenti-SceI-NG) which contains the SceI endonuclease (Taghian and Nickoloff, 1997). In these preliminary studies, DSB repair can be detected at 12h post-infection with Lenti-SceI-NG, and repair reaches plateau levels between 48h and 72h (**Figure 4, panel A**). To observe the effect of BRCC36 and BARD1 on HRR of DNA DSBs, we combined the pDR-GFP-*Sce*I system and siRNA approaches. We treated DR-GFP cells with either BRCC36 and BARD1 siRNAs or control siRNA twice (at 0 and 48h), and infected cells with SceI viral particles at

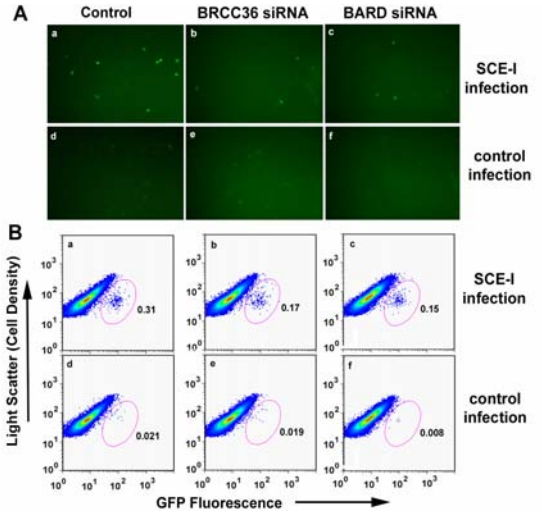

Figure 4. Depletion of BRCC36 Impairs Homologous Recombination Repair (HRR) in Cells Stably Transfected with the DR-GFP DNA Repair Substrate. A) Fluorescent microscopy of DR-GFP cells transduced with lentiviral vector expressing SCE-I restriction enzyme (*top panel)* or with control "gutted" lentivirus *(bottom panels)* at 72h post infection. Prior to infection, cells were transfected with siRNA-control (panels a & d), siRNA-BRCC36 (panels b & e), or siRNA-BARD1 (panels c & f) for 48 hours. B. Flow cytometry of DR-GFP cells at 72h post infection. GFP-positive cells were gated (in the circular insert with percentage indicated) according to two-dimensional plot of GFP-specific fluorescence versus light scatter to identify GFP-positive cells.

72h. Cells were collected for measuring the GFP signal at 72h after the infection. The results show that when BRCC36 and BARD1 expression were inhibited, the efficiency of HRR in these cells was lower than that in the control siRNA treated cells (**Figure 4, Panel A and B**), thus suggesting that BRCC36 and BARD1 are involved in HRR.

(3) ***To functionally characterize the role of BRCC36 in BRCA1-mediated ubquitination***

The BRCA1 N-terminal RING-finger region is the site of heterodimerization of BRCA1 and BARD1 (BRCAl-associated RING domain 1). When bound to BARD1, BRCA1 shows significant ubiquitin ligase activity and is capable of polymerizing ubiquitin. More importantly, deleterious mutations in the BRCA1 RING-finger domain abolish the ubiquitin ligase activity of BRCA1 (Hashizume, et al., 2001; Ruffner, et al., 2001). These findings suggest a relationship between BRCA1's ligase activity and the predisposition to breast cancer. At present, the known substrates that are polyubiquitinated by the BRCA1-BARD1 ubiquitin ligase are very limited. These known substrates include RNA polymerase II, nucleophosmin/B23, CtIP, and p53 (Dong, et al., 2003; Sato, et al., 2004; Starita, et al., 2004). We have previously reported that reconstitution of a recombinant four-subunit BRCC complex containing BRCA1/BARD1/BRCC45/BRCC36 reveals an enhanced E3 ligase activity compared to that of BRCA1/BARD1 heterodimer (Dong, et al., 2003). In addition, BRCC36 displays sequence homology with the human Poh1/Pad1 subunit of 26S proteasome and with the subunit 5 (Jab1) of COP9 signalosome (Dong, et al., 2003). The COP9 complex has been shown to regulate the activity of the SCF ubiquitin ligase complex (Lyapina, et al., 2001; Yang, et al., 2002). In collaboration with Dr. R. Dunbrack (Director, Molecular Modeling Facility at FCCC), we have performed protein modeling analysis on isoform B of BRCC36. A multiple-round PSI-BLAST sequence search was performed using the BRCC36 amino acid sequences. Suitable templates available in the Protein Data Bank (PDB) were chosen by using the PSIBLAST profiles obtained in the first step (Berman, et al., 2000). The backbone and the conserved parts of the template were copied from the PDB structure and amino acids different from the target sequence were built using SCWRL3 (Canutescu, et al., 2003). The molecular visualization and three-dimensional structure manipulation were performed using Chimera (Huang, et al., 1996). The data from the protein structure analysis suggest that BRCC36 contains a Jab1/MPN domain metalloenzyme (JAMM) motif, and the conserved BRCC36 residues (His122, His124, Asp135 and Ser132) form a catalytic site that binds a Zn atom (**Figure 5**). By analogy with the catalytic mechanism involved by cytidine deaminase, it is suggested that the Zn ion activates a water molecule that is subsequently involved in a nucleophilic attack in the process of isopeptide bond hydrolysis. Furthermore, JAMM motif has been found to have isopeptidase activity in the proteins involved in neddylation and ubiquitination (Cope, et al., 2002; Tran, et al., 2003). These findings from BRCC36 protein modeling suggest that BRCC36 may play a direct role in ubiquitination.

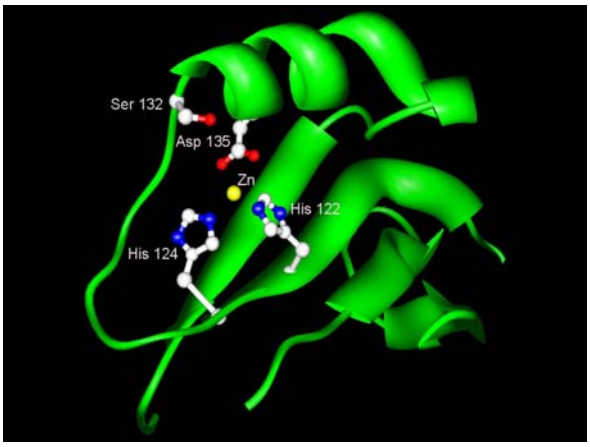

Figure 5. BRCC36 JAMM motif. Homology modeling procedures were used to obtain the three-dimensional model of isoform B of BRCC36. The molecular visualization and three-dimensional structure manipulation were performed using Chimera.

(4) ***Delivering BRCC36 into MEC lines using an electroporation expression system***

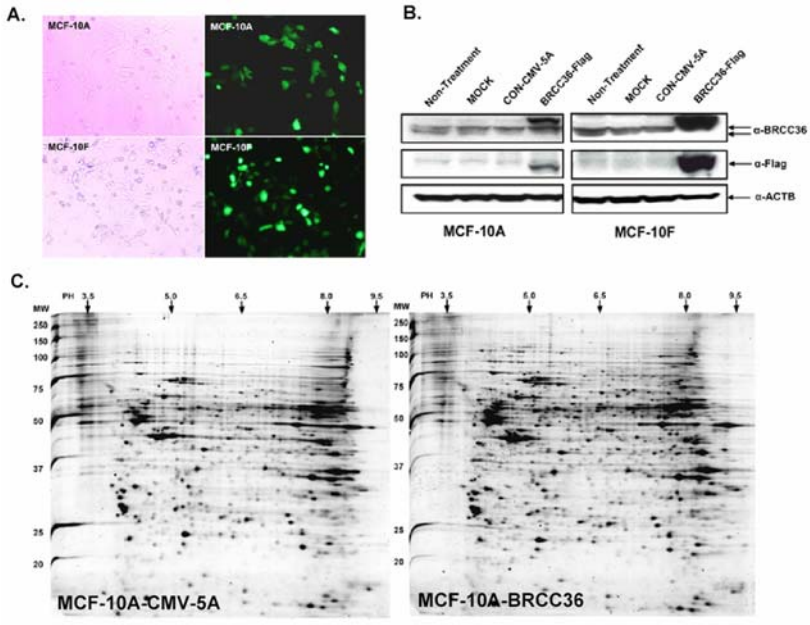

Fig. 6. Deliever BRCC36 into MEC lines. (**A**) One million MCF-10A or MCF-10F cells were resuspended in buffer provided in Nucleofector kit V (Amaxa), mixed with plasmid DNA (2μg) of pFLAG-CMV2-5a (empty vector) and pFLAG-CMV2-BRCC36, and electroporated with T-024 program of nucleofector (Amaxa) as suggested by the manufacturer. Transfection efficiency determined by eGFP is about 50% and 70% for MCF-10A and MCF-10F, respectively. (**B**) The expression of BRCC36 and Flag were determined by immunoblotting with specific antibodies. Protein loading levels were evaluated with anti-β-actin antibody. (**C**).Two-dimensional gels using protein lysates from containing MCF-10A cell transfected with CMV-5a empty vector (Left) and MCF-10A cell transfected with BRCC36-Flag (Right). Molecular weight markers are indicated in kDa and approximate isoelectric point is indicated across the top of the gels. These gels will provide sharp images for automated spot calling and image comparison by the progenesis software.

Our former studies have demonstrated that the expression of *BRCC36* mRNA is profoundly elevated in vast majority of breast tumors (Dong, et al., 2003). As I have hypothesized previously, aberrant expression of BRCCs, BRCA1-associated proteins, may create a BRCA1 "null" like phenotype. Germline *BRCA1* mutations have been found to be associated with a basal epithelial phenotype in breast cancer, and these basal-like breast cancers are typically diagnosed as high-grade invasive ductal carcinomas (Foulkes, et al., 2003). As a result of the highly aggressive phenotype of the cancer, BRCA1-associated breast cancer carriers often have a poor prognosis (Robson, 2000). Therefore, I propose to determine if aberrant expression of BRCC36 plays a role in the transformation of normal mammary epithelial cells (MECs). In this aspect, we have recently established an electroporation expression system using nucleofector technology (Amaxa). As shown in **Figure 6A**, transfection efficiency for this electroporation expression system determined by eGFP is about 50% and 70% for MCF-10A and MCF-10F, respectively. Results clearly show that BRCC36-Flag protein overexpressed in MEC lines transfected with pFlag-CMV-BRCC36 DNAs in comparing to empty vector controls (**Figure 6B**). We have also performed two-dimensional gels using protein lysates from containing MCF-10A cell transfected with CMV-5a empty vector or MCF-10A cell transfected with BRCC36-Flag (**Figure 6C**). These data will provide platform for further analysis of the insights of BRCC36 overexpression.

KEY RESEARCH ACCOMPLISHMENTS

1. Reported a frameshift *BRCC36* mutation (c.880insGGGGdup148) and a *CHEK2* c.1100delC) coexisted in the germline of a *BRCA1/2* mutation-negative breast cancer prone kindred;
2. Demonstrate that a frameshift *BRCC36* mutation (c.880insGGGGdup148) results in a stabilized mutant transcript in lymphoblastoid cell lines (LCLs) by immortalizing lymphocytes collected from the proband;

3. Demonstrated that silencing of BRCC36 decreased the expression of MRE11, a subunit of the R/M/N complex
4. Established a functional GFP-based assay to assess specific double-strand break repair, and demonstrated that depletion of BRCC36 impairs homologous recombination repair (HRR);
5. Demonstrated by protein structure analysis that BRCC36 contains a Jab1/MPN domain metalloenzyme (JAMM) motif, and the conserved BRCC36 residues form a catalytic site that binds a Zn atom. These findings from BRCC36 protein modeling suggest that BRCC36 may play a direct role in ubiquitination.
6. Succefully delivering BRCC36 into MEC lines using an electroporation expression system, and performing two-dimensional gels using protein lysates from containing MCF-10A cell transfected with CMV-5a empty vector or MCF-10A cell transfected with BRCC36-Flag.

REPORTABLE OUTCOMES

1. Abstracts

Chen, X., Arciero, C. A., Wang, C., Broccoli, D., and Godwin, A. K. Abrogation of BRCC36 enhances IR-induced apoptosis by disrupting BRCA1 activation and nuclear foci formation in breast cancer cells. In: Annual meeting of American Association of Cancer Research, (AB# 2305, Oral presentation), 2006.

Chen, X., Arciero, C. A., Wang, C., Broccoli, D., and Godwin, A. K. BRCC36 is Essential for IR-Induced BRCA1 Phosphorylation and Nuclear Foci Formation. In: Genome Instability and Repair, Keystone Symposia, 2007.

2. Publications

Chen, X., Arciero, C. A., Wang, C., Broccoli, D., and Godwin, A. K. BRCC36 is essential for IR-induced BRCA1 phosphorylation and nuclear foci formation. Cancer Res, 66: 5039-5046, 2006.

Chen, X., Truong, T.-T. N., Weaver, J., Bove, B. A., Cattie, K., Armstrong, B. A., Daly, M. B., and Godwin, A. K. Intronic alterations in BRCA1 and BRCA2: effect on mRNA splicing fidelity and expression. Human Mutation, 27: 427-35, 2006.

Chen, X., Arciero, C. A., and Godwin, A. K. BRCA1-associated complexes: new targets to overcome breast cancer radiation resistance. Expert Rev Anticancer Ther, 6: 187-196, 2006.

CONCLUSIONS

We have evaluated the role of BRCC36 in the ATM-BRCA1 DNA repair pathway in breast cancer cells in response to IR. The key findings of this work lie in the following: 1) depletion of BRCC36 enhances IR-induced apoptosis of breast cancer cells; 2) silencing of BRCC36 prevents the IR-induced activation of BRCA1 while other IR-response proteins, such as ATM, p53 and p21, are unaffected; 3) BRCC36 abrogation inhibits the formation of BRCA1 nuclear foci following IR, without preventing the interaction of BRCA1 with its well-characterized binding partner, e.g., BARD1; 4) depletion of BRCC36 impairs homologous recombination repair (HRR); and 5) *BRCC36* is mutated in the germline of a cancer-prone family and may increase the risk of developing breast cancer.

The damage caused by IR activates various DNA repair pathways, including the ATM/ATR/CHEK2 pathways (Cortez, et al., 1999; Waterman, et al., 1998). The central component of these DNA repair pathways is ATM kinase. ATM is activated by DNA damage and phosphorylates multiple factors, including BRCA1 and p53, which are involved in DNA repair, apoptosis and cell cycle arrest (Banin, et al., 1998; Canman, et al., 1998; Cortez, et al., 1999). As our results indicate, depletion of BRCC36 expression by RNAi blocks BRCA1 activation (i.e., phosphorylation and nuclear foci formation) in breast cancer cells following IR exposure. Because of the role of BRCA1 in DNA repair, I propose that an imbalance between the DNA repair/cell survival and DNA damage/cell apoptosis pathways exists in BRCC36-depleted cells following IR exposure. As a result, BRCC36 depletion appears to substantially sensitize breast cancer cells to IR-induced apoptosis (**Figure 7**). Overall, our studies define BRCC36 as a direct regulator of BRCA1 activation and nuclear foci formation in response to IR in a number of breast cancer cell lines. Therefore, aberrant expression or mutation of *BRCC36* genes in breast tumors may lead to disruption of the normal function of BRCA1 (i.e., leading to a BRCA1 null-like phenotype) and contribute to the development of breast cancer.

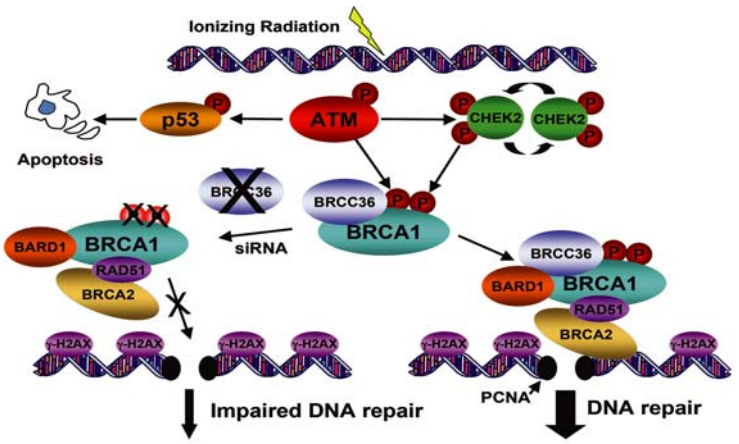

Figure 7. Model Illustrating the Potential Role of BRCC36 in the BRCA1-Associated DNA Repair Pathway in Response to IR.

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APPENDICES

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BRCC36 Is Essential for Ionizing Radiation–Induced BRCA1 Phosphorylation and Nuclear Foci Formation

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Abstract

We have previously reported the identification and characterization of a novel BRCA1/2 interacting protein complex, BRCC (BRCA1/2-containing complex). BRCC36, one of the proteins in BRCC, directly interacts with BRCA1, and regulates the ubiquitin E3 ligase activity of BRCC. Importantly, *BRCC36* is aberrantly expressed in the vast majority of breast tumors, indicating a potential role in the pathogenesis of this disease. To further elucidate the functional consequence of abnormal *BRCC36* expression in breast cancer, we have done *in vivo* silencing studies using small interfering RNAs targeting *BRCC36* in breast cancer cell lines, i.e., MCF-7, ZR-75-1, and T47D. Knock-down of *BRCC36* alone does not affect cell growth, but when combined with ionizing radiation (IR) exposure, it leads to an increase in the percentage of cells undergoing apoptosis when compared with the small interfering RNA control group in breast cancer cells. Immunoblot analysis shows that inhibition of BRCC36 has no effect on the activation of ATM, expression of p21 and p53, or BRCA1-BARD1 interaction following IR exposure. Importantly, BRCC36 depletion disrupts IR-induced phosphorylation of BRCA1. Immunofluorescent staining of BRCA1 and γ-H2AX indicates that BRCC36 depletion prevents the formation of BRCA1 nuclear foci in response to DNA damage in breast cancer cells. These results show that down-regulation of BRCC36 expression impairs the DNA repair pathway activated in response to IR by inhibiting BRCA1 activation, thereby sensitizing breast cancer cells to IR-induced apoptosis. (Cancer Res 2006; 66(10): 5039-46)

Introduction

Breast cancer is the most common cancer affecting women, with a woman's lifetime risk of breast cancer at ~10% by the age of 80 years. In the U.S., it was estimated that in 2005, ~211,000 new cases of breast cancer were diagnosed, and >40,000 deaths resulted from this disease (1). Breast cancer is a genetically heterogeneous disease, and germ line mutations in *BRCA1* and *BRCA2* genes predispose women to early onset breast cancer and/or ovarian cancer (2, 3). Since their cloning and characterization in the mid-1990s (4, 5), BRCA1 and BRCA2 proteins have been implicated in many cellular processes, including DNA repair and cell cycle-checkpoint control (6–10). BRCA1 has also been reported to be involved in protein ubiquitylation and chromatin remodeling (11, 12). Despite the fact that *BRCA1* and *BRCA2* mutations contribute to hereditary breast/ovarian cancer predisposition, somatic mutations are rarely found in sporadic breast cancers (13–15). Nevertheless, evidence is accumulating that dysfunction of other genes, coding for proteins in similar or redundant pathways as BRCA1 and BRCA2, might be important in the pathogenesis of a significant fraction of nonfamilial breast cancers. This speculation comes from several lines of evidence, including both phenotypic analyses of breast and ovarian tumors, as well as mechanistic studies of BRCA1- and BRCA2-associated pathways (16–18).

We have previously reported a novel multiprotein complex, termed BRCC, containing seven polypeptides including BRCA1, BRCA2, BARD1, and RAD51 (19). BRCC is an ubiquitin E3 ligase complex exhibiting an E2-dependent ubiquitination of the tumor suppressor p53. In this multiprotein complex, one of these proteins, referred to as BRCC36, has been found to be associated with BRCA1 and BRCA2, and has been shown to play an important role in the regulation of the ubiquitin E3 ligase activity of BRCC. The *BRCC36* gene is located at the *Xq28* locus, a chromosomal break point in patients with prolymphocytic T cell leukemia (20). BRCC36 displays sequence homology with the human Poh1/Pad1 subunit of the 26S proteasome and with subunit 5 (Jab1) of the COP9 signalosome (19). We have shown that cancer-associated mutations in BRCA1 abrogated the association of BRCC36 with BRCC and BRCA1 (19). Furthermore, reconstitution of a recombinant four-subunit BRCC complex containing BRCA1/BARD1/BRCC45/BRCC36 reveals an enhanced E3 ligase activity compared with that of BRCA1/BARD1 heterodimer (19). In addition, we have reported aberrant expression of *BRCC36* in the majority of breast cancer cell lines and invasive ductal carcinomas (19). The mechanism and consequences of abnormal *BRCC36* expression in breast cancer are presently unknown.

Previous studies have shown that BRCA1 is activated via the ATM/CHEK2 (CHK2) signaling pathway following the exposure of cells to DNA-damaging agents such as ionizing radiation (IR; refs. 21, 22). Following IR, BRCA1 is phosphorylated and forms discrete nuclear foci (dots) in response to DNA damage (23). Because BRCC36 directly interacts with BRCA1, we investigated the effects of knocking down BRCC36 expression, using small interfering RNAs (siRNA) on the growth and apoptosis of breast cancer cells. We further determined the role of BRCC36 in the BRCA1-associated DNA repair pathway activation following DNA damage. Here, our studies show that BRCC36 is a direct regulator of BRCA1 activation in response to IR.

Materials and Methods

Cell culture, siRNA transfection, and IR. Nontumorigenic epithelial cell lines, MCF-10F and 12A were purchased from American Type Culture Collection (Manassas, VA) and cultured in DMEM/F12 with reduced Ca^{2+} (0.04 mmol/L final), 20 ng/mL epidermal growth factor, 100 ng/mL cholera toxin, 0.01 mg/mL insulin, 500 ng/mL hydrocortisone, and 5%

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Chelex-treated horse serum. The human breast cancer cell lines, MCF-7, T47D, and ZR-75-1, were also obtained from the American Type Culture Collection. MCF-7 cells were maintained in DMEM medium, supplemented with 10% fetal bovine serum, penicillin, and streptomycin. T47D and ZR-75-1 cells were maintained in RPMI 1640, supplemented with 10% fetal bovine serum, penicillin, and streptomycin. 293-BARD1 cells were generously provided by Dr. R. Shiekhattar (Wistar Institute, Philadelphia, PA) and were maintained in MEM (Eagle) with 10% heat-inactivated horse serum.

For the *BRCC36* depletion studies, breast cancer cells were plated at a density of 5×10^3 cells/cm^2. After reaching 30% to 40% confluence, cells were transfected with siRNA using OligofectAMINE and OPTI-MEM I reduced serum medium (Invitrogen/Life Technologies, Inc., Carlsbad, CA) according to the manufacturer's protocol. The siRNA sequences targeting *BRCC36* corresponded to the coding region 253 to 273 bp (5′-AAGAG-GAAGGACCGAGTAGAA-3′) relative to the start codon. The corresponding siRNA duplexes with the following sense and antisense sequences were used: 5′-GAGGAAGGACCGAGUAGAAdTdT (sense) and 5′-UUCUACUCG-GUCCUUCCUCdTdT (antisense). This siRNA has been used in a previous study (19), as well as another siRNA targeting BRCC36 (corresponding to the coding region 120-138 bp). Both resulted in similar levels of transcript depletion. Green fluorescent protein siRNA was used as the negative control. All of the siRNA duplexes were synthesized by Dharmacon Research, Inc. (Lafayette, CO) using 2′-ACE protection chemistry. Twenty-four hours after the initial transfection, cells were subcultured and replated at 5×10^3 cells/cm^2. The cells were then retransfected under similar conditions 24 hours after replating. Seventy-two hours after the first transfection, the cells were irradiated using a Cesium 137 irradiator (model 81-14R). The cells received 4 Gy total IR (1.132 Gy/min for 3.53 minutes) for a targeted 50% induction of apoptosis (24). Cells were then grown for an additional 72 hours prior to harvesting and further analyses.

RNA isolation, reverse transcription, and quantitative PCR. Total cellular RNA was isolated from cells using TRIzol reagent (Invitrogen) according to the protocols provided by the manufacturer. Total RNA (2 μg) was used as a template to be reverse-transcribed in a 20 μL reaction containing 5 μmol/L random hexamers, 500 μmol/L of deoxynucleoside triphosphate mix, 1× reverse transcriptase buffer, 5 mmol/L $MgCl_2$, 1.5 units of RNase inhibitor, and 7.5 units of MuLV reverse transcriptase. All reagents were obtained from Applied Biosystems (Branchburg, NJ). The reaction conditions were as follows: 10 minutes at 25°C, 1 hour at 42°C, and 5 minutes at 94°C. The cDNA mixture (0.625 μL) was used in a real-time PCR reaction (25 μL total volume) done with ABI 7900HT (Applied Biosystems) following protocols recommended by the manufacturer. Optimal conditions were defined as: step 1, 95°C for 10 minutes; step 2, 95°C for 15 seconds, 60°C for 60 seconds with Optics, repeated for 40 cycles. The relative mRNA expressions of *BRCC36* were adjusted with β-actin (ACTB). The primer and probe sets used for real-time PCR were as follows: *BRCC36*, forward primer, 5′-AATTTCTCCAGAGCAGCTGTCTG; reverse primer, 5′-CATGGCTTGTGTGCGAACAT; TaqMan probe, (FAM) 5′-AACTGACAGGCCGCC-CCATGAG-(BHQ1); β-actin, forward primer, 5′-GCCAGGTCATCACCATTGG; reverse primer, 5′-GCGTACAGGTCTTTGCG-GAT; TaqMan probe, (Cal red) 5′-CGGTTCCGCTGCCCTGAGGC-(BHQ2).

Coimmunoprecipitation. 293 cells that stably express FLAG-BARD1 (19) at 70% to 80% confluence were washed twice with ice-cold D-PBS before scraping on ice with lysis buffer [50 mmol/L Tris-HCl (pH 7.4), with 150 mmol/L NaCl, 1 mmol/L EDTA, and 1% Triton X-100 and one tablet of protease inhibitor mixture per 40 mL of lysis buffer (Roche Molecular Biochemicals, Indianapolis, IN)]. Cellular debris was removed by centrifugation (14,000 × *g* for 15 minutes at 4°C), and protein concentrations were determined using the Bio-Rad detergent-compatible protein assay reagent. Cell lysate (1.5 mg) was added to the anti-FLAG M2-agarose affinity gel (Sigma, St. Louis, MO). All samples were placed on a roller shaker overnight at 4°C. After centrifugation, the supernatants were removed and the gel beads were washed thrice with 0.5 mL of wash buffer [50 mmol/L Tris-HCl (pH 7.4), with 150 mmol/L NaCl]. The beads were washed an additional four times with the wash buffer, resuspended in 20 μL of 2× SDS sample buffer before boiling for 5 minutes. Fifteen microliters of immunoprecipitate were separated by SDS-PAGE electrophoresis on 4% to 20% and 5% linear gradient Tris-HCl ready gels (Bio-Rad, Richmond, CA).

Western blot and antibodies. Cells were homogenized in lysis buffer [20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 2.5 mmol/L Na-pyrophosphate, 1 mmol/L Na-β-glycerophosphate, 20 mmol/L NaF, 1 mmol/L Na_3VO_4, 1 mmol/L phenylmethylsulfonyl fluoride, 1% Triton X-100, one tablet of protease inhibitor mixture (Roche Molecular Biochemicals)]. Cellular debris was removed by centrifugation as above. Protein concentrations were determined with Bio-Rad detergent-compatible protein assay (Bio-Rad). For BRCA1 and pATM, cells were lysed directly in Laemmli sample buffer (Bio-Rad). Proteins were resolved on 5% (BRCA1 and pATM) or 4% to 20% linear gradient (β-actin, p53, p21, and RAD51) SDS-PAGE ready gels at 120 V for 1.5 to 3 hours with 1× SDS running buffer. SDS-PAGE gels were transferred onto an Immobilon-P polyvinylidene difluoride membrane (Millipore, Bedford, MA). Primary antibodies used for Western analysis were mouse anti-BRCA1 (1:100; EMD Biosciences, San Diego, CA), mouse anti-phosphorylated ATM-S1981 (1:500; Rockland, Gilbertsville, PA), mouse anti-p53 (1:1,000, Cell Signaling, Beverly, MA), mouse anti-p21^{WAF} (1:100, EMD Biosciences), mouse anti-RAD51 (1:500; Upstate Biotechnology, Lake Placid, NY), mouse anti-FLAG M2 (1:1,000; Sigma), and mouse anti-β-actin (1:5,000; Sigma). Secondary antibodies were mouse and rabbit IgG, horseradish peroxidase–linked (1:10,000; Amersham, Piscataway, NJ). Perkin-Elmer Life Sciences renaissance enhanced luminol reagents (Boston, MA) were used as substrates for detection. To reprobe immunoblot membranes, Restore Western blot stripping buffer (Pierce, Rockford, IL) was used to strip the membrane.

Apoptosis assay. Breast cancer cells were collected by trypsinization and pelleted by centrifuging for 5 minutes at 800 × *g* at 4°C. After washing with 1 mL of ice-cold 1× Nexin buffer (Guava Technologies, Hayward, CA), the cells were resuspended in 100 μL of the same buffer. After labeling with Annexin V and 7-amino actinomycin D, the proportion of apoptotic cells was determined using a Guava personal cytometer (Guava Technologies) according to the manufacturer's instruction. Cell apoptosis was also analyzed using a terminal nucleotidyl transferase–mediated nick end labeling (TUNEL) assay. In brief, breast cancer cells were grown in four-well chamber slides (Nalge Nunc International, Rochester, NY). After fixing with 4% paraformaldehyde in PBS and permeabilizing with 0.1% Triton X-100 in 0.1% sodium citrate solution, apoptotic cells were detected using an *in situ* cell death detection kit (Roche, Germany) according to the manufacturer's instructions. At least 1,200 cells from eight independent fields were counted to evaluate the percentage of apoptotic cells.

Immunofluorescence and antibodies. MCF-7, ZR-75-1, and T47D cells were grown in four-well chamber slides (Nalge Nunc International) and processed for immunofluorescent analysis as described previously (25). For nuclear foci formation and colocalization of the BRCA1 and γ-H2AX, cells were preextracted in protein extraction solution (20 mmol/L HEPES, 50 mmol/L NaCl, 3 mmol/L $MgCl_2$, 300 mmol/L sucrose, and 0.5% Triton X-100), fixed in 3.7% formaldehyde (Fisher, Pittsburgh, PA) in PBS for 10 minutes and permeabilized in 0.5% NP40 in PBS prior to incubation with the following antibodies: rabbit anti-BRCA1 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) and mouse anti-γ-H2AX (1:200; Upstate Biotechnology). Primary antibodies were detected with tetramethyl rhodamine isothiocyanate–conjugated donkey anti-rabbit IgG and FITC-conjugated donkey anti-mouse IgG (1:100; Jackson ImmunoResearch, West Grove, PA). DNA was counterstained with 0.1 μg/mL of 4′,6′-diamidino-2-phenylindole (Sigma) and mounted in embedding medium (0.1% *p*-phenylene diamine in 90% glycerol, 1× PBS). Microscopic analysis was carried out using the Eclipse TE2000 (Nikon, Melville, NY) and images captured using a Cascade 650 monochrome camera (PhotoMetrics, Huntington Beach, CA). A series of 0.5-μm sections were collected for seven fields of each treatment group. Image acquisition from a Cascade 650 monochrome camera (PhotoMetrics) was controlled by MetaVue (v6.2r6, Universal Imaging/Molecular Devices, Downingtown, PA). An automated Ludl MAC2000 *x-y* stage and *z*-axis motor were also controlled using the MetaVue software (v6.2r6).

Image analysis. Quantification of BRCA1 and γ-H2AX nuclear foci formation was done with Metamorph software (v6.1; Universal Imaging/

Molecular Devices). In brief, a series of *Z*-sections for each channel was reassembled using the "maximum" type option within the "3-D reconstruction" function. Nuclei were defined in the 4′,6′-diamidino-2-phenylindole channel using the functions of "threshold for light objects" and "create regions from objects." Adjacent nuclei were separated into independent regions using the "cut-drawing" tool. Regions were then transposed onto the reassembled image for each digital channel, after background was removed using the "flatten background" function, and positive signals were identified by manual thresholding (high, 2,836; low, 1,758). For each nucleus, the number of the BRCA1 or γ-H2AX foci was calculated using the "foci measure" function. Approximately 70 cells of each treatment group from seven independent fields were analyzed to evaluate the number of BRCA1 foci.

Statistical analysis. Student's *t* test was employed using SAS software 8.0 (SAS Institute, Cary, NC). $P < 0.05$ was considered significant and results were presented as the mean ± SD.

Results

Inhibition of *BRCC36* gene expression by siRNA. To further elucidate the functional consequence of *BRCC36* aberrant expression in the pathogenesis of breast cancer, we did *in vivo* silencing studies targeting *BRCC36* in MCF-7, T47D, and ZR-75-1 breast cancer cell lines, which constitutively expresses high levels of *BRCC36* transcript relative to nontumorigenic breast epithelial cells, i.e., MCF-10F and MCF12A (Fig. 1*A*). Because antibodies specific to BRCC36 protein are not available, quantitative PCR was used to establish constitutive and attenuated *BRCC36* mRNA levels. We used siRNA targeting *BRCC36* or green fluorescent protein (negative control) to assess the response of BRCC36 depletion in breast cancer cells. Various siRNAs to BRCC36 were previously evaluated (19) and shown to be effective alone or in combination; however, for the purpose of these studies, *BRCC36*-siRNA1 was used. Treatment with this siRNA resulted in a >50% to 80% decrease in *BRCC36* mRNA levels in comparison to mock-treated or siRNA control–transfected cells ($P < 0.05$) by 72 hours. The greatest level of suppression was observed in MCF-7 cells (Fig. 1*B*).

Abrogation of *BRCC36*-enhanced IR-induced breast cancer cell apoptosis. Following depletion of *BRCC36* via siRNA, MCF-7, ZR-75-1, or T47D were treated with and without IR (4 Gy). The cells were cultured for an additional 72 hours prior to harvesting and were examined for DNA damage–induced cell apoptosis via Annexin V and 7-amino actinomycin D staining. No significant difference in the fraction of cells undergoing apoptosis in mock-treated, siRNA control-transfected, or siRNA-*BRCC36*–transfected cells was observed in the absence of IR, indicating that depletion of BRCC36 alone is not lethal (Fig. 2*A*; data not shown). However, when combined with *BRCC36* knock-down, IR exposure led to a significant increase in the percentage of MCF-7 cells that undergo apoptosis (45.9 ± 4.3%) when compared with the siRNA control group (34.9 ± 1.9%, $P < 0.05$; Fig. 2*A*; Table 1). Consistent with these results, the overall cell viability was substantially lower in siRNA-*BRCC36*–treated cells following IR as compared with control cells (50.9 ± 5.8% versus 58.4 ± 5.7%; Table 1). Similar results were observed in T47D cells treated with siRNA-BRCC36 and IR versus controls (42.2 ± 4.5% versus 23.3 ± 1.9%, $P < 0.05$; Fig. 2*A*; Table 1). Although the trend was evident for ZR-75-1 cells, the fraction of cells undergoing apoptosis following depletion of *BRCC36* and IR were not statistically significantly different (data not shown). Induction of apoptosis was confirmed using a TUNEL assay and MCF-7 cells (Fig. 2*B*). The combination of *BRCC36* siRNA abrogation and IR exposure again resulted in a significant increase in the fraction of cells undergoing apoptosis when compared with the siRNA control–treated cells (40.9 ± 2.7% versus 24.9 ± 3.3%, $P < 0.05$; Fig. 2*C*).

Inhibition of BRCC36 disrupted BRCA1 phosphorylation in breast cancer cells exposed to IR. Previous studies have indicated that the BRCA1 protein is phosphorylated in response to DNA-damaging agents (23). Because BRCC36 directly interacts with BRCA1 (19), we examined the effect of BRCC36 depletion on BRCA1-associated DNA repair/damage pathways. MCF-7 cells were treated with siRNA targeting *BRCC36*, and then exposed to 4 Gy of IR to induce DNA damage. MCF-7 cells were harvested 2 hours after IR. Western blot analysis was carried out to examine the expression and modification of BRCA1, p21, p53, and ATM. Western analysis clearly shows that DNA damage induced by IR resulted in increased expression of p21, stabilization of p53, and phosphorylation of BRCA1 and ATM (S1981) as expected (Fig. 3). This same pattern was observed in siRNA control–treated cells. Importantly, the reduction of BRCC36 blocked IR-induced phosphorylation of BRCA1. In comparison, BRCC36 knock-down had no effect on IR-induced expression of p21, stabilization of p53, and phosphorylation of ATM.

Effects of inhibition of BRCC36 on integrity of BRCA1-BARD1 heterodimer. To determine if depletion of BRCC36 affects the integrity of the BRCA1-BARD1 heterodimer, we targeted *BRCC36* mRNA in a 293-derived cell line expressing FLAG-BARD1

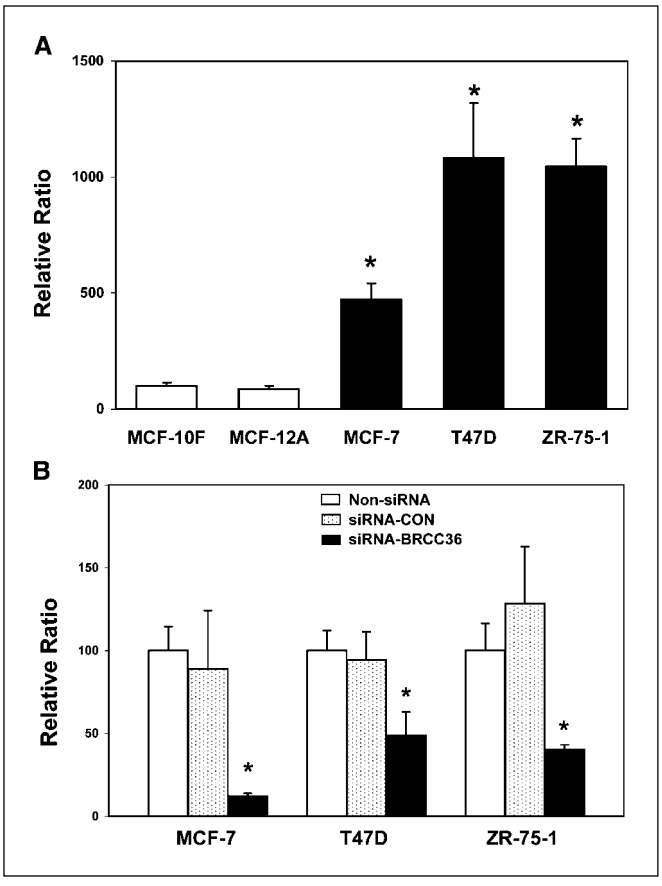

Figure 1. Abrogation of *BRCC36* expression by siRNA treatment. Quantitative reverse transcription-PCR analysis was done to examine the gene expression of *BRCC36*. The relative level of *BRCC36* expression was adjusted with β-actin (*ACTB*). *A*, *BRCC36* expression in nontumorigenic (*MCF-10F* and *MCF12A*) and tumorigenic (*MCF-7, ZR-75-1*, and *T47D*) breast epithelial cell lines. *B*, *BRCC36* expression in breast cancer cell lines after siRNA treatment ($P < 0.05$).

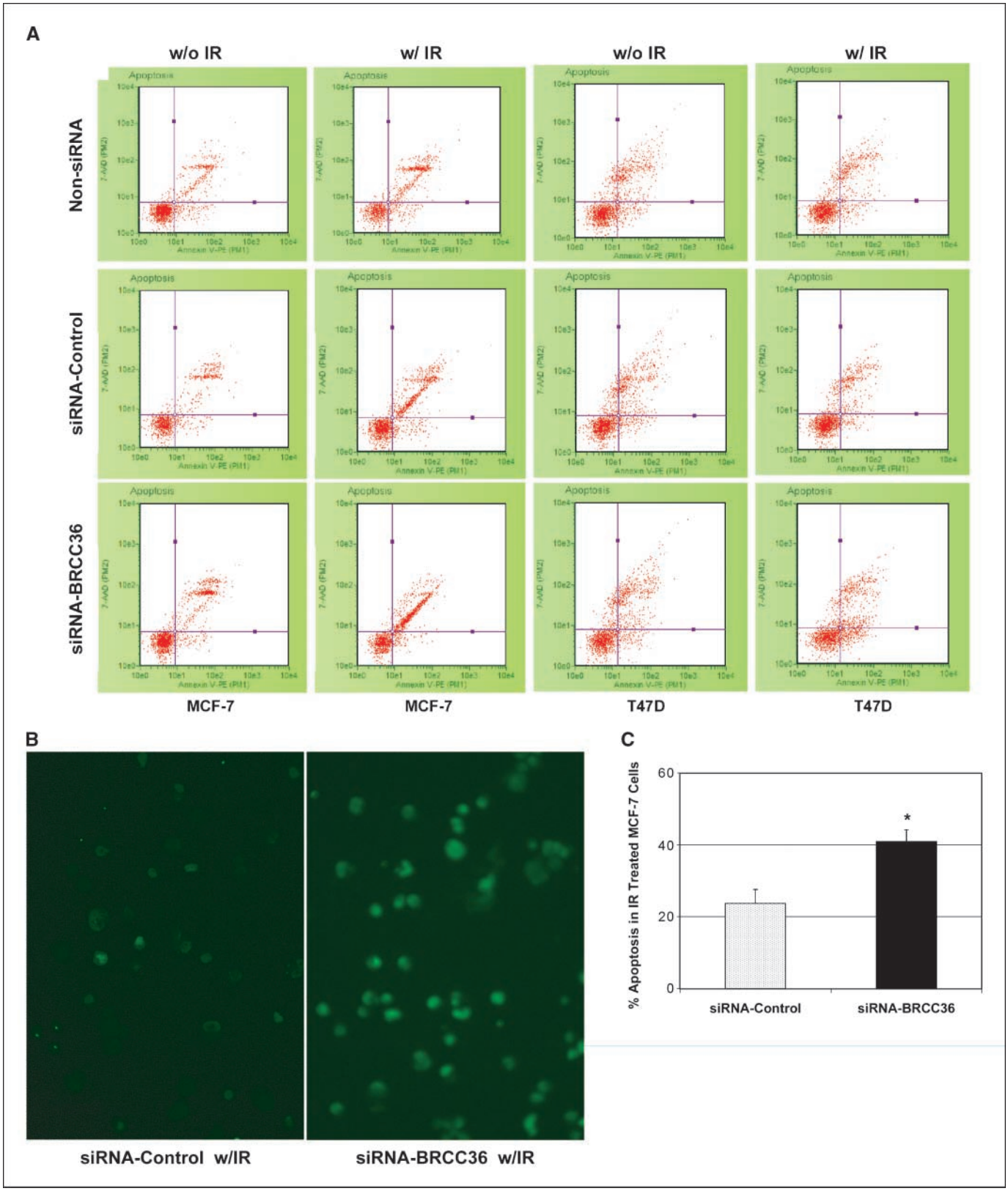

Figure 2. Apoptosis analysis in breast cells exposed to IR. *A,* MCF-7 and T47D cells were mock treated (*non-siRNA*) or were transfected with siRNA-control or siRNA-*BRCC36* prior to IR exposure. The proportion of apoptotic cells was measured following Annexin V and 7-amino actinomycin D staining using a Guava personal cytometer. All studies were done in triplicate. *B,* TUNEL labeling was done to detect apoptotic MCF-7 cells (*light green*) following exposure to IR. *C,* data analysis of the TUNEL assay. At least 1,200 cells of each treatment group from eight independent fields were counted to evaluate the percentage of apoptotic cells.

Table 1. Nexin assay in breast cancer cells exposed to IR

	MCF-7		T47D	
	Viable cells (%)	Apoptotic cells (%)	Viable cells (%)	Apoptotic cells (%)
Non-siRNA	57.3 ± 7.2	34.4 ± 4.5	68.5 ± 0.3	26.5 ± 0.4
siRNA-Control	58.4 ± 5.7	34.9 ± 1.9	70.5 ± 2.3	23.3 ± 1.9
siRNA-BRCC36	50.9 ± 5.8	45.9 ± 4.3*	52.7 ± 2.4	42.2 ± 4.5*

*siRNA-BRCC36 versus siRNA-Control (Student's t test, $P < 0.05$).

(19). Immunoprecipitation was done using anti-FLAG on lysates prepared from cells transfected with the BRCC36 or control siRNAs. BRCA1 and BARD1 were examined with SDS-PAGE and Western blot. Reduction of BRCC36 did not seem to alter the BRCA1-BARD1 interaction in either untreated or IR-treated cells (Fig. 4).

Inhibition of BRCC36 disrupts BRCA1 nuclear foci formation in breast cancer cells exposed to IR. It is well characterized that BRCA1 localizes to discrete nuclear foci (dots) during S phase or in response to DNA damage. In our previous report, we showed that BRCC36 directly interacts with BRCA1 at the region encompassing amino acids 502 to 1,054 (19). This region falls within the BRCA1 DNA-binding domain (amino acids 452-1079). Because the DNA-binding domain has been shown to contribute to the BRCA1 relocalization after DNA damage (23, 26), we sought to evaluate the role of BRCC36 in the formation of BRCA1 nuclear foci in response to DNA damage. MCF-7, T47D, and ZR-75-1 cells, mock-treated or transfected with siRNA-control or siRNA-*BRCC36*, were exposed to IR (4 Gy), and were evaluated for BRCA1 and γ-H2AX subcellular location. Recent studies have shown that the three breast cancer cell lines used in this study possess wild-type *BRCA1* (27). As shown in Fig. 5, BRCC36 deficiency inhibits BRCA1 focus formation as compared with mock-treated and siRNA control–transfected cells. Importantly, γ-H2AX response to IR was unaffected in the cells transfected with BRCC36 siRNA (Fig. 5*A-C*). Quantification of BRCA1 nuclear foci showed that siRNA-BRCC36 transfection in MCF-7 cells resulted in 63% and 52% decrease compared with siRNA-control cells at 2 and 4 hours post-IR, respectively ($P < 0.05$; Fig. 5*D* and *E*). Similar results were observed in T47D (49% and 36%) and ZR-75-1 (59% and 71%) cells (Fig. 5*D* and *E*). Collectively, these results show that down-regulation of BRCC36 expression impairs the DNA repair pathway activated in response to IR by inhibiting BRCA1 activation.

Discussion

In this study, we have evaluated the role of BRCC36 in the ATM/BRCA1 DNA repair pathway in breast cancer cells in response to IR. The key findings of this work lie in the following: first, we have shown that the depletion of *BRCC36* mRNA enhances IR-induced apoptosis of breast cancer cells (Fig. 2). Second, the reduction of BRCC36 prevents the IR-induced activation of BRCA1 whereas other IR response proteins, such as ATM, p53 and p21, are unaffected (Fig. 3). Third, BRCC36 abrogation inhibits the

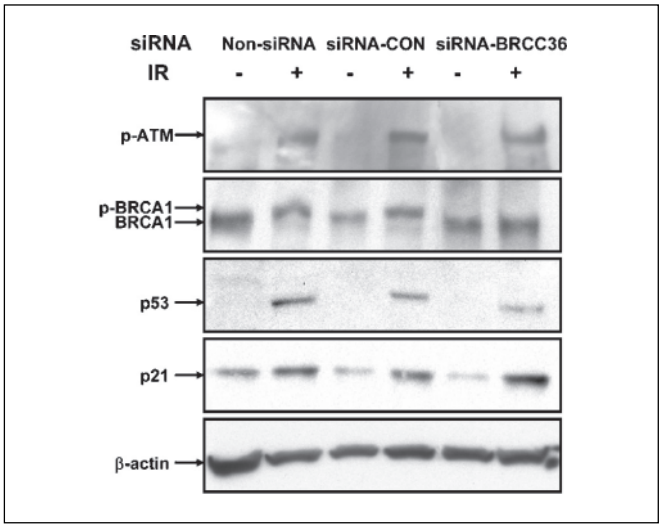

Figure 3. Activation of BRCA1 in response to IR treatment. MCF-7, MCF-7/siRNA control and MCF-7/siRNA-*BRCC36* cells were treated with or without IR (4 Gy), and cells were evaluated 2 hours after radiation exposure. BRCA1 protein was evaluated by immunoblotting with anti-BRCA1 antibody and shifts in the mobility of the protein bands indicated phosphorylated and unphosphorylated protein. The protein levels of phosphorylated ATM, p53, and p21 were determined by immunoblotting with anti-p-ATM, anti-p53, and anti-p21 antibodies, respectively. Protein loading levels were evaluated by immunoblotting with anti-β-actin antibody.

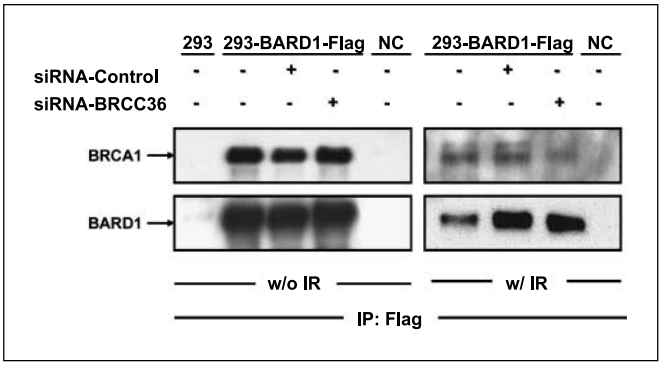

Figure 4. Effects of inhibition of BRCC36 on the integrity of BRCA1-BARD1 heterodimer. 293-BARD1-FLAG cells were transfected with either siRNA-*GFP* (*siRNA Control*) or siRNA-*BRCC36*. Transfected cells were then treated with 4 Gy IR and were incubated for 2 hours before harvesting. 293 cell lysate (1.5 mg; control or BRCC36-siRNA transfected) was incubated with ANTI-FLAG M2-agarose affinity gel. Immunoprecipitates were separated by SDS-PAGE electrophoresis. The protein levels of BRCA1 and BARD1 were determined by immunoblotting with anti-BRCA1 and anti-BARD1 antibody, respectively (*NC,* negative control).

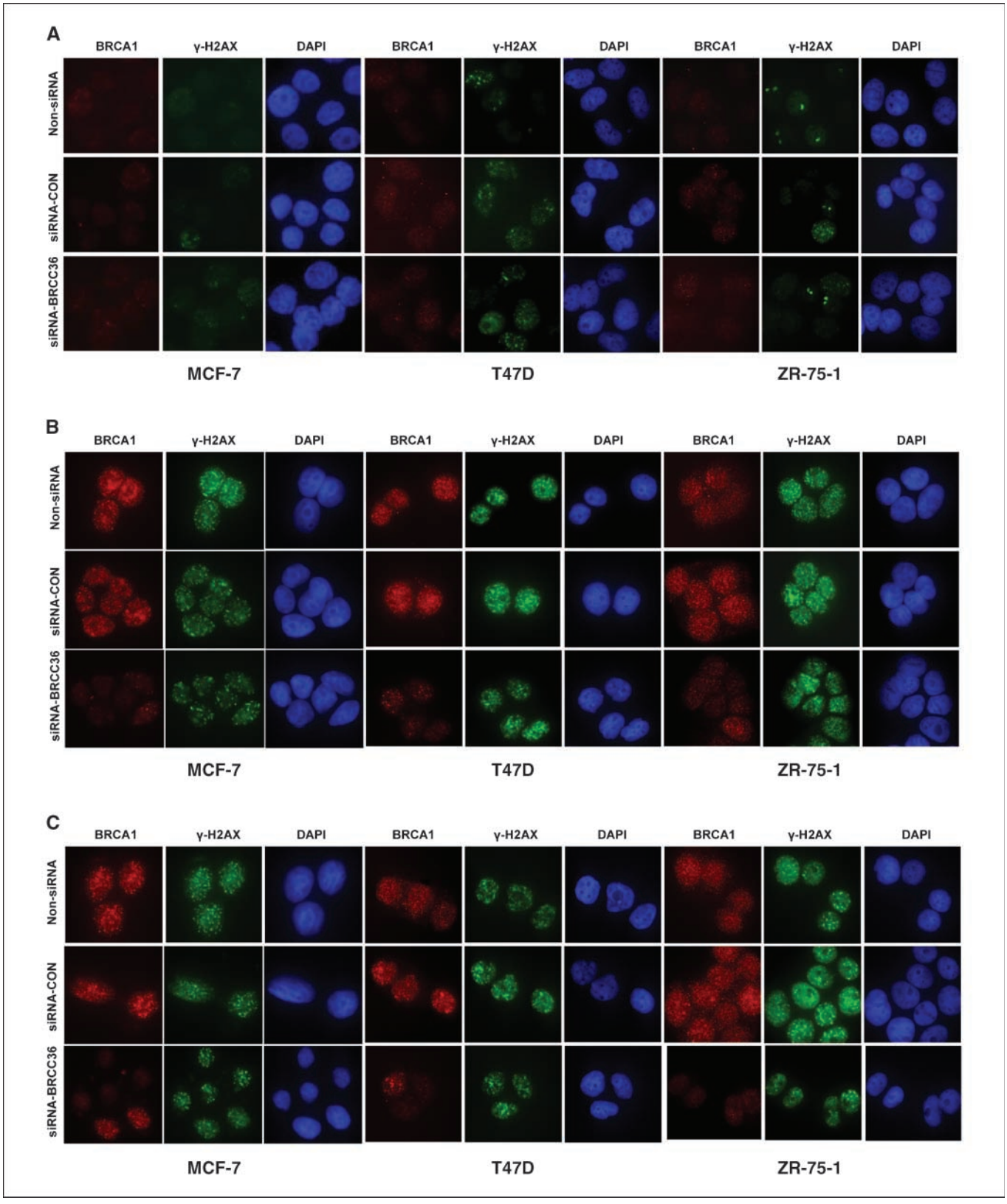

Figure 5. BRCA1 nuclear foci formation in breast cancer cells following IR exposure. MCF-7, ZR-75-1, and T47D cells were mock-treated (*non-siRNA*) or transfected with siRNA-CON or siRNA-BRCC36. Transfected cells were then treated with 4 Gy IR and were incubated for 2 or 4 additional hours. After pre-extraction and fixation, transfection cells then were immunostained for BRCA1 and γ-H2AX. Microscopic analysis was carried out using the Nikon Eclipse TE2000 and a Cascade 650 monochrome camera. Quantification of BRCA1 nuclear foci formation was done with Metamorph software (v6.1.). *A,* BRCA1 and γ-H2AX nuclear foci formation without IR exposure. *B,* BRCA1 and γ-H2AX nuclear foci formation at 2 hours post-IR exposure. *C,* BRCA1 and γ-H2AX nuclear foci formation at 4 hours post-IR exposure.

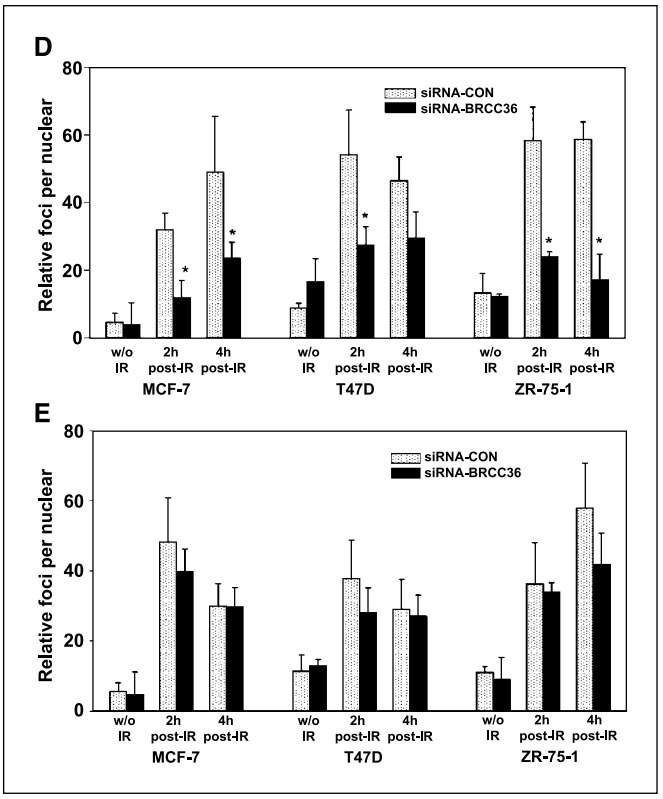

Figure 5 *Continued.* Quantification of BRCA1 (*D*) and γ-H2AX (*E*) nuclear foci formation without IR or at 2 and 4 hours post-IR exposure. Approximately 70 cells in each treatment group from seven independent fields were analyzed to evaluate the number of BRCA1 or γ-H2AX nuclear foci.

formation of BRCA1 nuclear foci following IR, without preventing the interaction of BRCA1 with its well-characterized binding partner, BARD1 (Figs. 4 and 5; data not shown).

The damage caused by IR activates various DNA repair pathways, including the ATM/ATR/CHEK2 pathways (21, 28). The central component of these DNA repair pathways is ATM kinase (29). ATM is activated by DNA damage and phosphorylates multiple factors, including BRCA1 and p53, which are involved in DNA repair, apoptosis and cell cycle arrest (21, 30, 31). As our results indicate, depletion of BRCC36 expression by siRNAi blocks BRCA1 activation, i.e., phosphorylation and nuclear foci formation in breast cancer cells following IR exposure, but has no direct effect on IR-induced apoptosis. Because of the role of BRCA1 in DNA repair, we propose that disrupting BRCA1 activation by BRCC36 depletion creates an imbalance between the DNA repair/cell survival and DNA damage/cell apoptosis pathways in cells following IR exposure (Fig. 6). As a result, BRCC36 depletion seems to substantially sensitize breast cancer cells to IR-induced apoptosis. However, it should be noted that these studies were done in a limited number of breast cancer cell lines, the caveat being that the DNA damage response may be altered in any or all cancer cell lines.

BRCA1 has been examined for a possible role in the development of radioresistant breast tumors. In fact, researchers have reported that BRCA1-deficient breast cancer cells have an increased sensitivity to IR (32). More recent studies have focused on the genes that code for proteins with equivalent/complementary functions to BRCA1 or function in the same pathway as BRCA1. A number of studies (33–37) have reported that manipulation of BRCA1-associated proteins affects cellular resistance or sensitivity to IR. The abnormal change (loss or gain) of any component in these BRCA1-related protein complexes may lead to their functional defects, which would result in a "BRCA1 null" phenotype. This may begin to explain why BRCA1 itself is rarely mutated and only occasionally (~10%) epigenetically down-regulated in sporadic diseases (18, 38). Therefore, BRCA1-associated proteins, including BRCC proteins, may serve as potential targets for the treatment of breast cancer, including radiation therapy.

In a previous report, we have shown that BRCC36 directly interacts with amino acids 502 to 1,054 of BRCA1 (19). In our current study, we have found that IR induced-BRCA1 nuclear foci formation is disrupted in BRCC36-depleted breast cancer cells (Fig. 5). The mechanism by which BRCC36 interferes with IR induced-BRCA1 localization is not clear. Previous studies have shown that BRCA1 consists of a DNA-binding domain region encompassing amino acids 452 to 1,079, and this BRCA1 DNA-binding domain contributes to the DNA repair–related functions of BRCA1, including the BRCA1 relocalization after DNA damage

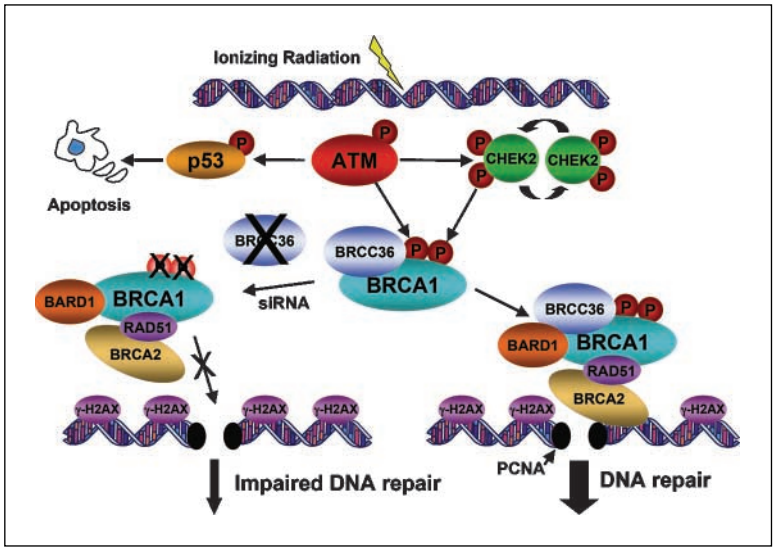

Figure 6. Model illustrating the potential role of BRCC36 in the BRCA1-associated DNA repair pathway in response to IR. BRCA1, p53, and CHEK2 are phosphorylated by ATM following DNA damage by IR; BRCA1 and p53 are involved in DNA repair and apoptosis, respectively. This activation leads to recruitment of many proteins to the site of the DNA damage, including BARD1, RAD51, BRCA2, and presumably BRCC36. Depletion of BRCC36 via siRNAs prevents the phosphorylation of BRCA1 and disrupts BRCA1 nuclear foci formation following IR, whereas γ-H2AX remains associated with regions of DNA damage. Due to the role of BRCA1 in DNA repair, the balance between the DNA repair/cell survival and DNA damage/cell apoptosis is disrupted and depletion of BRCC36 therefore sensitizes breast cancer cells to IR-induced apoptosis.

(23). The function of BRCA1 DNA-binding domain has been reported to be partially mediated through a protein complex, termed as BRCA1-associated surveillance complex (BASC; ref. 26). Interestingly, the location of BRCA1 DNA-binding domain coincides with the region that BRCC36 binds to, i.e., amino acids 452 to 1,079 versus amino acids 502 to 1,054 of BRCA1, respectively. In this study, we have found that depletion of BRCC36 by siRNA knock-down prevents the phosphorylation of BRCA1 following IR (Fig. 3). Previous studies have shown that BRCA1 is bound and phosphorylated by the ATM kinase and the G_2-M control kinase (CHEK2) after IR (21, 39, 40). Coincidentally, a host of studies have suggested that ATM and CHEK2 also bind to this central region of BRCA1 [reviewed by Narod and Foulkes (38)]. These findings may provide insight as to why depletion of BRCC36 in our studies inhibits BRCA1 activation, e.g., BRCC36 could help recruit BRCA1 to ATM and CHEK2 or stabilize their interactions following activation of the DNA damage response pathway. Our future studies are geared towards determining if BRCC36 remains associated with activated BRCA1 or whether BRCC36 must be displaced prior to phosphorylation by ATM and CHEK2. We have begun to explore these questions and have found that BARD1 and RAD51 remain associated with BRCA1 following BRCC36 depletion (Fig. 4; data not shown). However, we have yet to determine if BRCC36 depletion affects the interaction between BRCA1 and ATM and/or CHEK2 (data not shown).

Overall, our studies define BRCC36 as a direct regulator of BRCA1 activation and nuclear foci formation in response to IR in a number of breast cancer cell lines. Our results suggest that down-regulation of BRCC36 expression impairs the DNA repair pathway activated in response to IR and seems to sensitize breast cancer cells to IR-induced apoptosis. Therefore, it is intriguing to speculate that targeting BRCC36 may aid in the treatment of radiation-resistant breast tumors.

Acknowledgments

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Review

BRCA1-associated complexes: new targets to overcome breast cancer radiation resistance

Xiaowei Chen†, Cletus A Arciero and Andrew K Godwin

Since BRCA1 was cloned a decade ago, significant progress has been made in defining its biochemical and biological functions, as well as its role in breast and ovarian cancers. BRCA1 has been implicated in many cellular processes, including DNA repair, cell cycle checkpoint control, protein ubiquitination and chromatin remodeling. This review examines the role(s) of BRCA1 in mediating these cellular processes, and discusses its potential involvement in the resistance of breast cancer to radiation-based therapies. Finally, the possibility that BRCA1-associated proteins may serve as new targets for breast cancer radiation therapy is explored. The activation or inactivation of these BRCA1-associated proteins may modify both the risk of developing cancers in *BRCA1* mutation carriers and the efficacy of breast cancer therapy, including radiation.

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CONTENTS

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BRCA1, breast cancer, cell cycle, chromatin remodeling, DNA repair, protein interaction, radiation resistance, ubiquitination

Breast cancer is the most common cancer affecting women, with a lifetime risk of approximately 10% by the age of 80 years. In the USA, it is estimated that in 2005 there was approximately 211,000 new cases of breast cancer, and more than 40,000 breast cancer-related deaths [1]. Inherited mutations in the breast cancer susceptibility gene (*BRCA1*) and *BRCA2* predispose women to high risks of breast and ovarian cancer [2]. Lifetime risks of breast cancer are as high as 80% among American women with mutations in these genes; lifetime risks of ovarian cancer are more than 40% for *BRCA1* and 20% for *BRCA2* mutation carriers [2]. Risks for young women with inherited *BRCA1* or *BRCA2* mutations are particularly increasing. Among Caucasian women in the USA, between 5 and 10% of breast and ovarian cancer cases are due to inherited mutations in *BRCA1* and *BRCA2* [3,4]. Mutations in other genes also influence breast cancer risk, including *CHEK2*, *TP53* (associated with Li–Fraumeni syndrome) and *PTEN* (associated with Cowden syndrome) [5,6].

Since its cloning and characterization in the mid-1990s [7], BRCA1 has been implicated in many cellular processes including DNA repair, cell cycle checkpoint control, protein ubiquitination and chromatin remodeling. Despite the fact that *BRCA1* mutations contribute to hereditary breast and ovarian cancer, it is surprising that *BRCA1* is rarely found to be mutated in sporadic breast cancers, which account for approximately 90% of all breast cancers [8]. Nevertheless, studies have indicated that loss of *BRCA1* expression through epigenetic mechanisms may contribute significantly to sporadic breast cancer [9,10]. In addition, evidence is accumulating that dysfunction of other genes coding for proteins in complementary pathways to BRCA1, might be important in the pathogenesis of a significant proportion of sporadic, nonfamilial cancers. This speculation comes from several lines of evidence, including both phenotypic analyses of breast and ovarian tumors as well as mechanistic studies of BRCA1-associated pathways [11,12].

BRCA1 has been implicated in normal cellular processes, including DNA fidelity and damage repair, and has therefore been examined as having a possible role in the radioresistance of breast tumors. Breast cancer cell line and animal model studies have shown an increased sensitivity to ionizing radiation when BRCA1 is defective [13–17]. This review

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will provide an update on what is currently known about the molecular and cellular functions of BRCA1, and speculate on how these functions contribute to the pathogenesis of breast cancer.

BRCA1: a breast cancer susceptibility gene

Genetic transmission of an autosomal dominant factor responsible for familial breast carcinoma was first reported in the early 1970s [18]. Hall and colleagues made significant progress in breast cancer molecular genetics during the 1990s by utilizing gene linkage studies, which identified an association between a locus on chromosome 17q21 and early onset familial breast cancer [19]. *BRCA1* was isolated in 1994 and is composed of 23 exons (22 of which are coding) that are distributed over approximately 80 kbp of genomic DNA (GenBank reference: NM_007295.2 and NC_000017.9). The 7.4-kb transcript is detected in numerous tissues, including breast and ovary, and encodes a predicted protein of 1863 amino acids. Many alternatively spliced transcript variants have been reported for *BRCA1*, but the significance of these in regards to function is not well known as only a few code for a protein that has been detected and studied functionally. Nevertheless, several groups have reported that some of the alternative spliced forms of *BRCA1* may influence its subcellular localization, as well as its ability to interact with other proteins, and thus, its physiological functions [20,21]. Interestingly, BRCA1 shows little homology to other known proteins except for 42 amino acids at the amino terminus that encode a really interesting new gene (RING)-finger, a motif found in a subset of proteins that interact with nucleic acids and/or form protein–protein complexes [22]. In addition, a region of BRCA1 encoded by exon 11 (amino acids 200–300) possesses a nuclear localization signal and sequences that serve as docking sites for many critical cellular proteins [23]. Furthermore, BRCA1 contains two BRCA1 C-terminal (BRCT) domains in its C-terminal region (amino acids 1650–1860) (FIGURE 1) [24]. The BRCT motif of BRCA1 binds to many proteins, including RNA polymerase (RNAP) II, p300, BACH1, p53 and retinoblastoma (RB), and plays an important role in DNA repair and/or cell cycle checkpoints [24].

Since the identification of *BRCA1*, there has been a tremendous amount of effort focusing on the genetic characterization of this gene and the related culprit breast susceptibility gene, *BRCA2*. As a result of mutation screening that has spanned the globe and all nationalities afflicted with hereditary breast and ovarian cancer, more than 1500 sequence variants [101] have been detected throughout *BRCA1* (FIGURE 1). In spite of the large number of sequence variants in *BRCA1*, only frameshift mutations (insertions/deletions, some of which can be several kbp in length), splice site mutations (that lead to aberrant mRNA splicing and disrupt the generation of the normal protein product), nonsense mutations (single bp substitutions that result in premature termination of protein translation and, therefore, expression of a truncated protein) and a few missense changes (a single bp exchange that results in an amino acid substitution) are accepted as disease-associated genetic alterations [25,26]. Little research has been performed regarding other types of mutations, primarily intron variants and missense changes in coding sequence, in order to investigate their potential roles in the predispositions to breast and ovarian cancers [27,28]. These genetic alterations are therefore typically classified as variants of unknown/uncertain significance (VUS). These VUSs pose a major clinical challenge in the management of patients from cancer-prone families, since an 'indeterminate' result frequently leaves both the genetic counselors and patients searching for more answers. Currently, few of these VUSs have been proven experimentally to be deleterious and disease causing. It is likely that the vast majority of VUSs will ultimately be proven to be benign polymorphisms or variants associated with a modest increase in cancer risk.

Function of *BRCA1*: a puzzle not fully solved

BRCA1 is one of the most intensively studied genes in the breast cancer research field due to its clinical importance. The *BRCA1* gene encodes for a 220-kDa nuclear phosphoprotein that has been suggested to play a role in maintaining genomic stability and to act as a tumor suppressor. BRCA1 interacts directly or indirectly with other tumor suppressors (such as p53 and BRCA2), DNA damage sensors (such as RAD51, RAD50, MRE11 and NBS1) and signal transducers (such as p21 and cyclin B) to form multisubunit protein complexes, such as

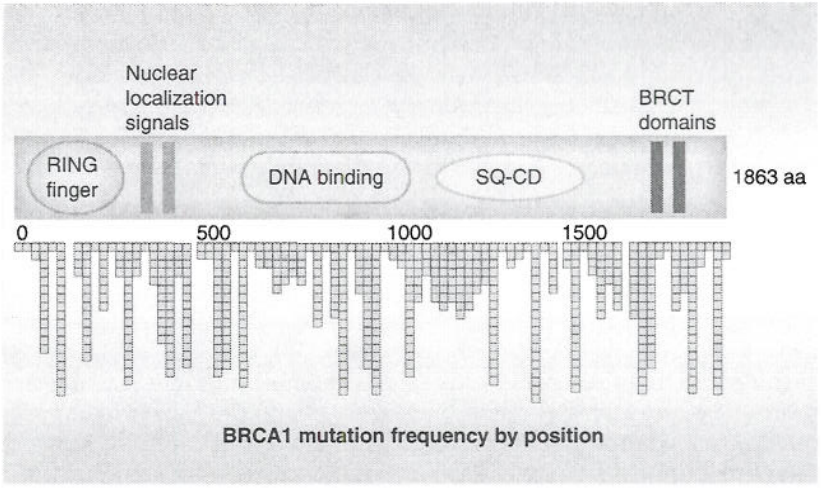

Figure 1. Schematic of the functional domains of BRCA1 and mutation frequency graph. The *BRCA1* gene codes a protein with 1863 amino acids and five protein functional domains. The five domains are the RING finger, nuclear localization signal, DNA binding domain, SQ (serine and threonine)-cluster domain (CD) and BRCA1 carboxyl terminus (BRCT) domain. The height of the vertical bar presents the relative mutation frequency across the BRCA1 protein. [Modified from Breast Cancer Information Core (BIC) database].

BRCA1-associated genome surveillance complex (BASC) and BRCA1 and BRCA2 containing complex (BRCC). These multisubunit protein complexes are involved in a broad range of biological processes including DNA repair, cell cycle control, ubiquitination and chromatin remodeling. However, the number of these BRCA1 protein-associated complexes and their complexity has yet to be fully elucidated. Thus, much of the current scientific effort involving BRCA1 is being directed at defining the biochemical functions of BRCA1 and its protein interactions.

BRCA1 cellular localization

The BRCA1 protein undergoes hyperphosphorylation during late G_1 and S phases of the cell cycle [29]. The nuclear localization signal motif (amino acids 200–300) appears to serve as a docking site for importin-α, a subunit of the nuclear transport signal receptor [23]. This physical interaction has led to the speculation that BRCA1 may be involved in the nuclear localization sequence (NLS) receptor-mediated pathway of nuclear import and play a role in recruiting other proteins to the DNA double-strand break (DSB) site following DNA damage [30]. In proliferating cells, BRCA1 forms discrete nuclear foci (dots) during the S phase as well as the G_2 phase of the cell cycle. Loss of the BRCA1 foci in subsequent phases of the cell cycle is accompanied by a specific, dose-dependent change in the state of BRCA1 phosphorylation [31]. Following cellular exposure to ionizing radiation, UV or various chemotherapeutic agents, such as cisplatin, BRCA1 becomes phosphorylated and forms discrete nuclear foci in response to DNA damage [31]. These observations continue to indicate that phosphorylation of BRCA1 is a critical step for dynamic BRCA1 nuclear localization during cell proliferation and the cellular response to DNA damage.

Role of BRCA1 in DNA repair

The majority of BRCA1 functional studies have focused on its potential role in response to DNA damage. The direct implication of BRCA1 as a component of DNA damage response pathways comes from its interactions with BRCA2 and RAD51. BRCA1, BRCA2 and RAD51 form a protein complex that activates DSB repair and initiates homologous recombination, which links the maintenance of genomic integrity to tumor suppression [32]. In addition, Scully and colleagues found that cells expressing exogenous wild type BRCA1 were less sensitive to γ-irradiation and were more efficient in repairing DSBs compared with a BRCA1-deficient breast cancer line, HCC1937 [33]. In animal models, impaired repair of chromosomal DSBs by homologous recombination have been noted in BRCA1-deficient mouse embryonic stem cells [34]. Furthermore, increased levels of chromosomal damage have been reported in lymphocytes following irradiation exposure in *BRCA1* mutation carriers [35]. Recent studies indicate that BRCA1 is also involved in nonhomologous end joining. *BRCA1* mutants make this error-prone repair mechanism even less accurate, which could lead to chromosomal rearrangement and instability [36,37]. In addition, BRCA1 has been shown to have a role in nucleotide-excision repair. BRCA1 specifically enhances the global genomic repair (GGR) pathway by inducing the expression of nucleotide excision repair (NER) genes, including XPC, DDB2 and GADD45, independent of p53 [38].

Role of BRCA1 in cell cycle control

BRCA1 has been shown to stimulate expression of the cyclin-dependent kinase (CDK) inhibitor, p21, and to inhibit cell cycle progression into S phase [39]. In addition, researchers have shown that BRCA1 is not only essential for activating the CHEK1 kinase that regulates G_2/M arrest induced by DNA damage, but also controls the expression, phosphorylation and cellular localization of Cdc25C and Cdc2/cyclin B kinases [40]. Therefore, BRCA1 appears to be involved in regulating the onset of mitosis. In mouse knockout models where the *BRCA1* gene is mutated in such a way that BRCA1 protein is not functional, $BRCA1^{-/-}$ (null) embryos fail to fully develop. However, if either *Tp53* or *p21* are also eliminated by genetic approaches, *BRCA1* mutant embryos ($BRCA1^{-/-}$; $Tp53^{-/-}$ or $BRCA1^{-/-}$; $p21^{-/-}$) show prolonged survival from embryonic days 7.5 to 9.5 [41]. In addition, a defective G_2/M checkpoint and extensive chromosomal abnormalities have been found in cells from other *BRCA1* knockout mice in which exon 11 was removed [42]. It is also reported that elimination of one *Tp53* allele ($Tp53^{+/-}$; $BRCA1^{exon11-/-}$) rescued this embryonic lethality caused by the deletion of *BRCA1* exon 11 and restored normal mammary gland development [43]. However, most female mice homozygous for the *BRCA1* exon 11 deletion and heterozygous for loss of the *Tp53* gene developed mammary tumors within 6–12 months. Importantly, the resulting tumors lost the remaining *Tp53* allele [43]. These findings indicate that the genetic interactions between *BRCA1* and *p53* are associated with breast carcinogenesis. Moreover, primary fibroblasts, which are heterozygous for a *BRCA1* mutation, display an abnormal G_1/S cell cycle checkpoint following UVA irradiation [44].

Role of BRCA1 in ubiquitination

The RING-finger motif in the N-terminus of BRCA1 has been found to be the site of heterodimerization of BRCA1 and BRCA1-associated RING domain 1 (BARD1). BARD1 is another RING-finger-containing protein that was identified using the BRCA1 RING-finger domain as the 'bait' in a protein interaction assay (yeast two-hybrid screen) [45]. When bound to BARD1, BRCA1 shows significant ubiquitin ligase activity and is capable of polymerizing ubiquitin [46]. Importantly, deleterious mutations affecting the BRCA1 RING-finger domain, found in clinical specimens, abolish the ubiquitin ligase activity of BRCA1 [16,45]. These findings support a relationship between BRCA1's ligase activity and a predisposition to breast cancer.

BRCA1 interacts with a number of proteins and functions in diverse cellular processes. Therefore, it is not surprising that BRCA1's ubiquitin ligase activity may contribute to more than one of the biological roles of BRCA1. BRCA1 has also been reported to interact with the RNAP II holoenzyme [47]. Two recent reports have suggested that the BRCA1/BARD1 complex may be involved in the degradation of RNAP complex,

and siRNA-mediated knockdown of BRCA1 and BARD1 results in stabilization of RNAP II in the cells following UV exposure [48,49]. These studies reported that BRCA1/BARD1 appears to initiate the degradation of stalled RNAP II and, thus, disrupt the coupled transcription by inhibiting RNA processing machinery in cells exposed to DNA damage. At present, the known substrates that are polyubiquitinated by the BRCA1–BARD1 ubiquitin ligase are very limited and include RNAP II, nucleophosmin/B23 and p53 [48–51].

Role of BRCA1 in chromatin remodeling

Wang and colleagues used immunoprecipitation and mass spectrometry to identify a large multisubunit protein complex, BASC, in which the DNA repair proteins identified include ATM, BLM, MSH2, MSH6, MLH1, the RAD50–MRE11–NBS1 complex and the RFC1–RFC2–RFC4 complex [52]. Confocal microscopy demonstrated that BRCA1, BLM and the RAD50–MRE11–NBS1 complex colocalized to large nuclear foci, and BASC has subsequently been shown to be involved in chromatin remodeling at sites of DSBs [52]. In addition, BRCA1 directly interacts with the brahma-related gene 1 (BRG1) subunit of SW1/SNF-associated complex, which is involved in chromatin remodeling [53]. This finding links chromatin remodeling processes to breast cancer. Furthermore, the BRCT domain of BRCA1 has been reported to be associated with the histone deacetylases, HDAC1 and HDAC2 [54]. All of these findings help explain the involvement of BRCA1 in multiple, seemingly unrelated processes such as transcription and DNA repair.

BRCA1-associated proteins: targets for breast cancer radiation therapy

In the past several decades, efforts have been made toward understanding the mechanisms of the response to both cytotoxic chemotherapy and ionizing radiation breast cancer treatment. Owing to the important role of BRCA1 in DNA repair, cell cycle control and other pathways, breast tumors with defective BRCA1 are likely to be more sensitive to chemotherapy and ionizing radiation. Although both chemotherapy and radiation have been extensively studied in the clinical treatment of breast cancer, the following section will explore the possibility that BRCA1 and its interacting proteins could become novel targets in enhancing breast cancer radiation therapy. The role of BRCA1 in chemotherapy has been reviewed comprehensively by other groups [55,56].

Radiation therapy for breast cancer

Radiation therapy has been a treatment modality for breast cancer patients for more than 100 years. Over the past 30 years, radiation therapy has become a critical step in the successful treatment of breast cancer. The role of radiation therapy grew in the early 1970s, when Fletcher documented its instrumental role in decreasing local recurrences [57]. In particular, supraclavicular metastases were reduced from 20–25% to 1.3–3% with the addition of ionizing radiation. Radiation therapy was also used to treat patients with tumors that had undergone total mastectomy, producing a decrease in local recurrences of over two-thirds [57]. This early work led to an expanded role for radiation therapy in breast cancer.

The emergence of radiation therapy to the forefront of modern breast cancer treatment lies in its application in breast-conservation therapy. Landmark studies on the necessity of radiation therapy in breast-conservation therapy were performed by Fisher and colleagues, as a part of the National Surgical Adjuvant Breast and Bowel Project (NASBP) B-06 trial, which demonstrated that lumpectomy with radiation therapy had much lower recurrence rates than lumpectomy alone (14 vs 39%) [58]. This observation has been further validated by an extensive meta-analysis that supported improved local control with the addition of radiation therapy [59,60].

Recently, clinical research has examined the possible survival benefits of radiation therapy in breast cancer. The Early Breast Cancer Trialists' Collaborative Group (EBCTG) examined 78 trials involving more than 42,000 patients with breast cancer [61]. In the analyses of trials directly comparing patients receiving radiation therapy with those not receiving radiation therapy, a clear reduction in local recurrences was again seen in the radiotherapy group, including patients undergoing mastectomy or breast-conservation therapy [61]. Interestingly, there was also a noted improvement in survival in patients treated with radiotherapy. In fact, patients receiving radiotherapy for breast cancer had a 5.4% reduction in their 15-year breast cancer mortality risk and a 4.4–5.3% reduction in overall mortality [61]. These findings support the observation that radiotherapy contributes to not only the reduction of local recurrences, but also the reduction in 15-year overall mortality.

Despite the benefits of radiation therapy in the treatment of breast cancer, patients still develop local recurrences in the targeted breast. Researchers have noted that breast cancer recurrences in the nonirradiated breast often occur within 3 years of initial diagnosis [62]. In comparison, local recurrences in irradiated breast tissue occur much later, and the risk increases with time (7, 14 and 20% risk at 5, 10 and 20 years, respectively) [63]. It is these recurrences that have spurred research in both breast cancer recurrences and the possibility of radioresistant breast tumors.

BRCA1 in resistance to breast cancer resistance therapy

The damage caused by ionizing radiation activates DNA damage cell cycle checkpoints leading to the various DNA repair pathways. The central component of these pathways is the ATM/CHEK2 kinase. ATM/CHEK2 is activated by DNA damage before phosphorylating multiple proteins, including BRCA1 [64–66]. BRCA1 is phosphorylated at tyrosine residues by ATM, the gene mutated in ataxia telangiectasia, or by ATM and Rad-3-related (ATR) kinase in response to ionizing radiation-induced DNA damage [65,67]. In addition to ATM and ATR, BRCA1 is also phosphorylated by CHEK2, the human homolog of yeast checkpoint protein kinase (hCds1), in response to ionizing radiation-induced DNA damage [66]. This

phosphorylation occurs in a region that contains clusters of serine–glutamine residues, and is functionally important as shown by mouse studies with mutated *BRCA1* that lack these phosphorylation sites. These mice fail to rescue the radiation hypersensitivity when introduced into BRCA1-deficient cells [65]. In addition, BRCA1 phosphorylation by ATM/CHEK2 following DNA damage is critical for recruiting BRCA1 to the DNA repair and chromatin remodeling protein complexes [68].

The potential role of BRCA1 in radioresistant breast cancer has been examined. Studies using breast cancer cell lines report an increased sensitivity to ionizing radiation when *BRCA1* is mutated [13–17]. However, studies of breast cancer patients fail to reliably replicate these *in vivo* findings [69–71]. A recent study found that *BRCA1* mutation carriers exhibit increased sensitivity to radiation therapy by examining the rate of breast cancer recurrence following breast-conserving treatment [72]. However, Pierce and colleagues conducted a multicenter study of *BRCA1* mutation carriers and noted no significant difference between *BRCA1* mutation carriers and women with sporadic breast cancer, in terms of local recurrences [73]. Two additional studies indicated that mutations in *BRCA1* may not account for clinical radiation hypersensitivity [69,71]. These conflicting findings pose the question of whether *BRCA1* mutations will indeed increase the sensitivities of tumor cells to radiation-based therapies. Therefore, the role of BRCA1 and its influence on tumor cell sensitivity to radiation *in vivo* and *in vitro* will require further investigation.

Proteins in BRCA1-associated complexes

Mutations in *BRCA1* may not be the only way to inhibit its activity and, thus, increase cancer susceptibility and sensitivity to radiation-based therapies. There is growing evidence suggesting that disruption of the BRCA1-associated complex, either through mutations or aberrant expression of a key member or members of these multiprotein complexes, may result in loss of normal activity. Significant changes in the stoichiometry of proteins within these complexes may lead to their inactivation, which, in turn, would result in a BRCA1 null-like phenotype. In addition, proteins both upstream of BRCA1, such as ATM, ATR and CHEK2, and downstream, such as p53, RB and CHEK1, when altered, may prevent appropriate activation of BRCA1 or transmission of signals initiated by BRCA1 functional complexes, respectively (FIGURE 2). This may help to explain why BRCA1 itself is not frequently mutated in sporadic disease. BRCA1-associated proteins may, therefore, become potential targets for the treatment of breast cancer, including radiation therapy. A number of studies have reported that manipulation of BRCA1-associated proteins has an impact on cellular resistance or sensitivity to ionizing radiation (TABLE 1) [70,74–83].

In this aspect, a novel multiprotein complex, termed BRCC, containing seven polypeptides, including BRCA1, BRCA2, BARD1 and RAD51, has recently been identified [50]. BRCC is an ubiquitin E3 ligase complex that can cause E2-dependent ubiquitination of the tumor suppressor, p53. In this multiprotein complex, one protein, referred to as BRCC36, is associated with BRCA1 and BRCA2 and plays an important role in the regulation of the ubiquitin E3 ligase activity of BRCC. BRCC36 displays sequence homology with the human Poh1/Pad1 subunit of the 26S proteasome, and with subunit five (Jab1) of the COP9 signalosome. Cancer-associated mutations in *BRCA1* abrogate the association of BRCC36 with BRCC. Reconstitution of a recombinant four-subunit BRCC complex, BRCA1/BARD1/BRCC45/BRCC36, revealed an enhanced E3 ligase activity compared with that of the BRCA1/BARD1 heterodimer [50]. In addition, the authors have reported a profound increase in *BRCC36* expression in the majority of breast cancer cell lines and clinical breast tumors [50]. Importantly, depletion of BRCC36 enhances apoptosis in breast cancer cells following ionizing radiation by preventing the

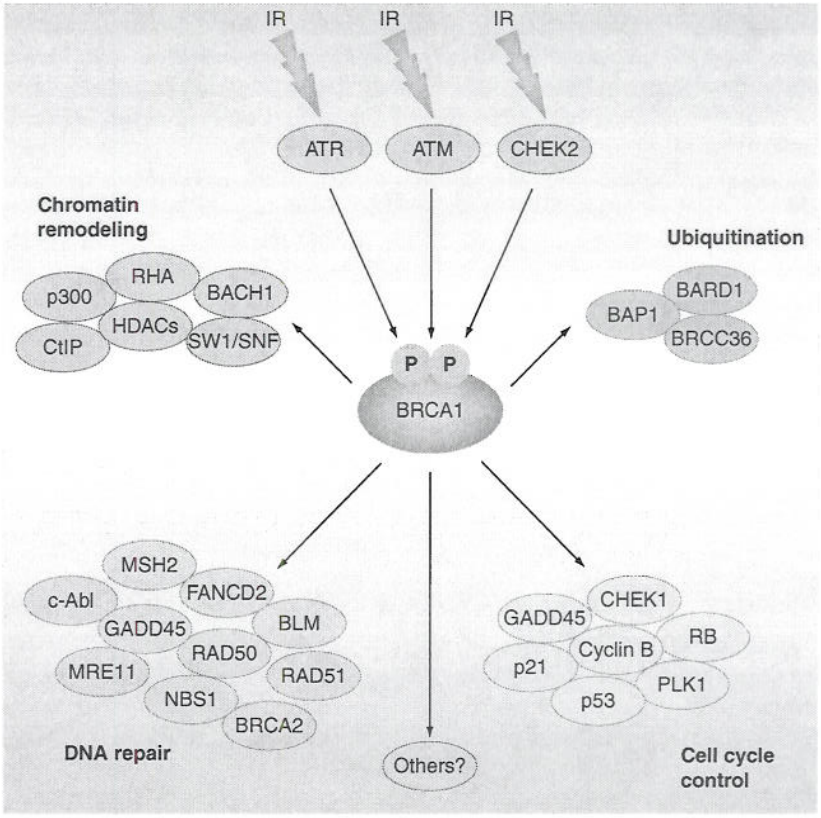

Figure 2. BRCA1-associated protein network. BRCA1 interacts with a number of proteins to form multisubunit protein complexes. BRCA1-associated protein complexes are involved in DNA repair, cell cycle checkpoint control, protein ubiquitination and chromatin remodeling.
BARD1: BRCA1-associated RING domain; IR: Ionizing radiation; BRCA1: Breast cancer susceptibility gene 1.

Table 1. Radiation sensitivity studies related to the BRCA1-associated proteins.

Protein name	BRCA1 pathway	Manipulation approach	Increase resistance	Increase sensitivity
FANCD2	DNA repair	Defected		Garcia-Higuera et al., 2001 Houghtaling et al., 2005
NBS1	DNA repair	Defected		Nakanishi et al., 2002
MRE11	DNA repair	Disrupted		Digweed et al., 2002
RAD51	DNA repair	Deficiency Blocking Overexpression	 Vispe et al., 1998	Lio et al., 2004 Russell et al., 2003
HDAC	Chromatin remodeling	Blocking		Chinnaivan et al., 2005
RB	Check point control	Decreasing Defected		Carlson et al., 2000 Billecke et al., 2002
BARD1	Ubiquitination	Depleted		Boulton et al., 2004
BRCC36	Ubiquitination	Depleted		Dong et al., 2003

BARD: BRCA1-associated RING domain; BRCC: BRCA1 and BRCA2 containing complex.

phosphorylation (i.e., activation) of BRCA1 and by disrupting the BRCA1 nuclear foci formation following IR [CHEN ET AL., UNPUBLISHED OBSERVATIONS]. Based on the role of BRCA1 in DNA repair, the authors propose that disrupting activation of BRCA1 by BRCC36 depletion will create an imbalance between the DNA repair/cell survival and cell apoptosis/death pathways in cells following IR exposure (FIGURE 3). As a result, BRCC36 depletion is likely to substantially sensitize breast tumor cells to IR-induced apoptosis. Due to the fact that the BRCC36 protein has been found aberrantly expressed in the majority of breast cancers and is a major regulator of BRCA1 function, it may serve as a therapeutic target in the management of radiation-resistant breast tumors. Since these findings are mainly based on *in vitro* studies, further examination of the *in vivo* influence of these protein targets on cell sensitivity to radiation is warranted.

Expert commentary

In the last decade since *BRCA1* was cloned, significant progress has been made in defining its biochemical and biological functions and its role in breast and ovarian cancers. Mutations in *BRCA1* have been established to predispose women to breast and ovarian cancer. The BRCA1 protein is also involved in the cellular processes of DNA repair, gene transcription, ubiquitination and chromatin modification. However, little is known about the mechanism by which BRCA1 modulates these processes. Extensive studies have shown that BRCA1 interacts with a number of regulatory proteins to form functional complexes. The activation or inactivation of these BRCA1-associated

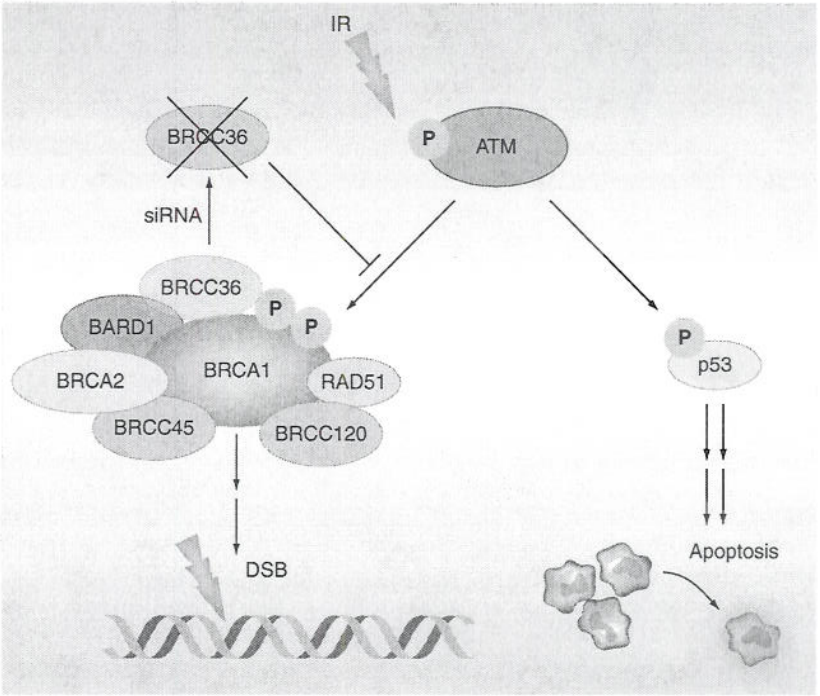

Figure 3. A proposed model illustrating the role of BRCC36 in the BRCA1-associated DNA repair pathway in response to ionizing radiation. BRCA1 and p53 are phosphorylated by ATM induced by IR, and are involved in DNA repair and apoptosis, respectively. Depletion of BRCC36 prevents the phosphorylation of BRCA1 and disrupts the BRCA1 nuclear foci formation in breast cancer cells following ionizing radiation. Based on the role of BRCA1 in DNA repair, the authors propose that disrupting activation of BRCA1 by BRCC36 depletion will create an imbalance between the DNA repair/cell survival and cell apoptosis/death pathways in cells, following exposure to ionizing radiation. As a result, abrogation of BRCC36 appears to sensitize breast cancer cells to IR-induced apoptosis.
ATM: Ataxia telangiectasia-mutated gene; BARD: BRCA1-associated RING domain; BRCC: BRCA1 and BRCA2 containing complex; DSB: Double strand break; IR: Ionizing radiation.

proteins may modify both the risk of developing cancers in *BRCA1* mutation carriers and the efficacy of breast cancer therapy, including radiation therapy.

Five-year view

Further efforts are needed to clarify, in detail, the mechanisms by which BRCA1 collaborates with other key proteins and the molecular pathway through which it is regulated. It will also be helpful to explore the role of other factors that associate with BRCA1, such as BRCA2, RAD51, BARD1, BAP1, BRCC proteins and a potential host of others. These studies may help uncover the range of biological and biochemical functions of BRCA1 and its associated proteins, in order to better elucidate their role in the pathogenesis of breast and ovarian cancer. Only then will it be possible to intelligently design molecular-targeted therapies that may selectively attack these cancer cells.

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Key issues

- Breast cancer susceptibility gene 1 (*BRCA1*) was cloned in 1994 and encodes a 220-kDa nuclear phosphoprotein (1863 amino acids).
- Among thousands of genetic alterations detected in *BRCA1*, more than 700 are deleterious germline mutations that are associated with an increased risk of breast and ovarian cancer.
- The lifetime risk of cancer among female *BRCA1* mutation carriers is estimated to be as high as 80% for breast cancer and more than 40% for ovarian cancer.
- BRCA1 interacts with a number of proteins to form multisubunit protein complexes.
- The number of these protein complexes and their complexity has yet to be fully elucidated.
- BRCA1-associated protein complexes are involved in DNA repair, cell cycle checkpoint control, protein ubiquitination and chromatin remodeling.
- The complete spectrum of the biochemical functions of BRCA1 and its interactors is not fully understood.
- Despite the benefits of radiation therapy in the treatment of breast cancer, patients still develop local recurrences in the targeted breast.
- BRCA1-deficient breast cancer cell lines show increased sensitivity to ionizing radiation. However, studies of breast cancer *BRCA1* carriers fail to reliably replicate these findings *in vivo*.
- A number of studies have reported that manipulation of BRCA1-associated proteins impacts cellular resistance or sensitivity to ionizing radiation.
- Significant changes in the stoichiometry of proteins within these complexes may lead to their inactivation, which, in turn, would result in a BRCA1 null-like phenotype.
- BRCA1-associated proteins may serve as potential therapeutic targets in treating familial and sporadic forms of breast cancer and in enhancing the efficacy of conventional therapies, including radiation-based approaches.

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Website

101 Breast Cancer Information Core (BIC) http://research.nhgri.nih.gov/bic/

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RESEARCH ARTICLE

Intronic Alterations in *BRCA1* and *BRCA2*: Effect on mRNA Splicing Fidelity and Expression

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Germline mutations in the human breast cancer susceptibility genes *BRCA1* and *BRCA2* account for the majority of hereditary breast and ovarian cancer. In spite of the large number of sequence variants identified in *BRCA1* and *BRCA2* mutation analyses, many of these genetic alterations are still classified as variants of unknown significance (VUS). In this study, we evaluated 12 *BRCA1/2* intronic variants in order to differentiate their pathogenic or polymorphic effects on the mRNA splicing process. We detected the existence of aberrant splicing in three *BRCA1* variants (c.301-2delA/IVS6-2delA, c.441+1G>A/IVS7+1G>A, and c.4986+6T>G/IVS16+6T>G) and two *BRCA2* variants (c.8487+1G>A/IVS19+1G>A and c.8632-2A>G/IVS20-2A>G). All but one of the aberrant transcripts arise from mutations affecting the conserved splice acceptor or donor sequences and all would be predicted to result in expression of truncated BRCA1 or BRCA2 proteins. However, we demonstrated that four of these splice-site mutations (i.e., c.301-2delA, c.441+1G>A, c.4986+6T>G, and c.8632-2A>G) with premature termination codons were highly unstable and were unlikely to encode for abundant expression of a mutant protein. Three variants of *BRCA1* (c.212+3A>G/IVS5+3A>G, c.593+8A>G/IVS9+8A>G, and c.4986-20A>G/IVS16-20A>G) and four variants of *BRCA2* (c.516-19C>T/IVS6-19C>T, c.7976-4_7976_3delTT/IVS17-4delTT, c.8487+19A>G/IVS19+19A>G, and c.9256-18C>A/IVS24-18C>A) in our studies show no effects on the normal splicing process, and they are considered to be benign polymorphic alterations. Our studies help to clarify the aberrant splicing in *BRCA1* and *BRCA2* as well as provide information that can be used clinically to help counsel breast/ovarian cancer prone families. Hum Mutat 27(5), 427–435, 2006. Published 2006 Wiley-Liss, Inc.†

KEY WORDS: BRCA1; BRCA2; splicing; nonsense-mediated mRNA decay; NMD; variants of unknown significance; breast cancer; ovarian cancer

INTRODUCTION

Germline mutations in human breast cancer susceptibility genes, *BRCA1* (MIM# 113705) and *BRCA2* (MIM# 600185), are responsible for the vast majority of hereditary breast and ovarian cancer [Bove et al., 2002; Ford et al., 1998]. The lifetime risk of cancer among female *BRCA1* or *BRCA2* mutation carriers is estimated to be between 36 and 85% for breast cancer and between 16 and 60% for ovarian cancer [Breast Cancer Linkage Consortium, 1999; King et al., 2003; Malone et al., 2000]. To date, approximately 3,400 sequence variants (listed online at the Breast Cancer Information Core [BIC]; http://research.nhgri.nih.gov/bic) in each of these genes have been identified by extensive mutational analyses since their cloning and characterization in the mid-1990s [Frank et al., 2002; Miki et al., 1994; Wooster et al., 1995]. However, in spite of the large number of sequence variants in *BRCA1* and *BRCA2*, only frameshifts (insertions/deletions), splice-sites, nonsense mutations, and a few missense mutations are accepted as disease-associated genetic alterations [Breast Cancer Linkage Consortium, 1999; Frank et al., 1998; Shattuck-Eidens et al., 1997]. Little has been done with respect to other types of mutations, including intronic variants, to investigate their possible roles in the predisposition to breast and ovarian cancer [Arnold et al., 2002]. These genetic alterations, therefore, are typically classified as variants of unknown/uncertain significance (VUS). These VUSs pose a clinical challenge in the management of patients from cancer-prone families. Because of their unknown functional significance, the medical management of individuals in these families remains a dilemma.

Among these VUSs, alterations occurring in intron-exon boundaries of *BRCA1* and *BRCA2* have potential impacts on splicing fidelity. A previous study has demonstrated that variants

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at the splicing sites affecting mRNA processing account for 15% of point mutations that associate with the genetic diseases [Krawczak et al., 1992]. Approximately 5% of all *BRCA1* and *BRCA2* alterations are splice-site mutations (http://research.nhgri.nih.gov/bic). However, only a few of these mutations have been examined for their effects on *BRCA1* and *BRCA2* mRNA splicing fidelity [Agata et al., 2003; Brose et al., 2004; Campos et al., 2003; Claes et al., 2003; Fetzer et al., 1999; Keaton et al., 2003; Laskie Ostrow et al., 2001; Pyne et al., 2000; Scholl et al., 1999] and few if any have been evaluated for their effect on the stability of the mutant transcripts.

Alterations within splicing sites of *BRCA1* and *BRCA2* also have potential impacts on mRNA transcript stability, because the mutant allele caused by aberrant splicing may contain a premature termination codon (PTC) which could activate the nonsense-mediated mRNA decay (NMD) pathway. NMD represents a phylogenetically widely conserved splicing- and translation-dependent mechanism that eliminates transcripts with PTCs and suppresses the accumulation of C-terminally truncated peptides [Frischmeyer and Dietz, 1999; Weischenfeldt et al., 2005]. Elimination of frameshift transcripts that result from aberrant splicing has been suggested as an important function of NMD [Frischmeyer and Dietz, 1999; Weischenfeldt et al., 2005]. Blocking NMD with translation inhibitor, e.g., puromycin, has been used to help detect aberrant spliced alleles which contain PTCs [Andreutti-Zaugg et al., 1997; Nomura et al., 2000].

As a result of mutation screening of *BRCA1* and *BRCA2* in hundreds of breast cancer prone kindreds, we identified six *BRCA1* and six *BRCA2* intronic alterations that are located at or near an exon-intron boundary. The purpose of this study was to characterize these 12 *BRCA1/2* variants in order to differentiate their pathogenic or polymorphic effects on mRNA splicing and expression. Our studies further defined the consequence of these sequence variants on RNA splicing and provided information regarding stability of the various mutant transcripts.

MATERIALS AND METHODS

Nomenclature and Databases

Both the Human Genetic Variation Society (HGVS) approved guidelines (www.hgvs.org/mutnomen) and the BIC (Breast Cancer Information Core; http://research.nhgri.nih.gov/bic) traditional system have been used in our studies for *BRCA1* and *BRCA2* nomenclature. In order to achieve the accuracy and also facilitate the published data comparison, an alteration was expressed as "HGVS approved/BIC Traditional" when it first appeared in the article. RefSeqs (GenBank accession no. NM_007295.2 and NM_000059.1) have been used for HGVS-approved *BRCA1* and *BRCA2* DNA and mRNA numbering, respectively. RefSeqs (GenBank accession no. NP_009226.1 and NP_000050.1) have been used for HGVS-approved BRCA1 and BRCA2 amino acid numbering, respectively. The A of the ATG translation initiation codon is +1, according to approved guidelines. RefSeqs (GenBank accession no. U14680 and U43746) have been used for BIC traditional *BRCA1* and *BRCA2* numbering, respectively.

Family Risk Assessment Program (FRAP) and *BRCA1* and *BRCA2* Mutation Analysis

Women with a family history of breast and ovarian cancer who were enrolled in the Family Risk Assessment Program (FRAP) were eligible for the present study. The FRAP was designed to provide education and cancer risk counseling for women with at least one first-degree relative (i.e., mother, sister, or daughter) with breast or ovarian cancer. Blood samples were collected following informed consent for participating in various FRAP-directed research studies including genetic susceptibility testing. Constitutional DNAs isolated from 375 participants' lymphocytes were screened for mutations in *BRCA1* and *BRCA2* as previously described [Andrulis et al., 2002; Kulinski et al., 2000; Oleykowski et al., 1998].

Lymphoblastoid Cell Lines (LCL)

Lymphocytes isolated from FRAP blood samples were infected with Epstein-Barr virus (EBV) to establish the immortal lymphoblastoid cell lines (LCLs). LCLs were maintained in RPMI media (GIBCO BRL, Gaithersburg, MD; www.gibcobrl.com) supplemented with 20% fetal calf serum (FCS) and antibiotics at 37°C, 5% CO_2 atmospheric conditions, and 95% humidity. The immortalized LCLs from cancer-free individuals who had tested negative for mutations in *BRCA1* and *BRCA2* served as wild-type controls. To prevent potential degradation of unstable transcripts by nonsense-mediated mRNA decay, a translational inhibitor, puromycin (Sigma, St. Louis, MO; www.sigmaaldrich.com), was added to the EBV cells at the concentration of 200 μg/ml for 14 hr before total RNAs were isolated.

RNA Isolation and RT-PCR

Total cellular RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA; www.invitrogen.com), according to the protocols provided by the manufacturer (www.invitrogen.com/content/sfs/manuals/10296010.pdf). Purified RNA samples were further processed with DNA-free kit as suggested by the manufacturer (Ambion, Houston, TX; www.ambion.com). After quantification with a Bioanalyzer-2100 system using RNA 6000 Nano LabChip kits (Agilent Technologies, Palo Alto, CA; www.agilent.com), 2 μg of total RNA from each sample was used as a template to be reverse-transcribed (RT) in a 20-μl reaction, which also contained 5 μM random hexamers, 500 μM deoxynucleoside triphosphate mix, 1× reverse transcriptase buffer, 5 mM $MgCl_2$, 1.5 units of RNase inhibitor, and 7.5 units of MuLV RTase. All reagents were purchased from Applied Biosystems (Branchburg, NJ; www.appliedbiosystems.com). The reaction conditions were 10 min at 25°C, 1 hr at 42°C, and 5 min at 94°C. Then, 2 μl of the cDNA mixture was used in a PCR reaction, with specific primers targeting the potential splicing site. Optimal conditions were defined as: Step 1, 95°C for 10 min; Step 2, 94°C for 15 sec, 60°C for 30 sec, 72°C for 45 sec, repeated for 40 cycles; Step 3, 72°C for 7 min. The detailed information with regard to primer design and sequences are available upon request. The products were electrophoresed on 2% agarose gels. Uncloned PCR fragments were cleaned with QIAquick PCR Purification Kit (Qiagen, Hilden, Germany; www.qiagen.com), sequenced and analyzed in both sense and antisense directions for the presence of heterozygous splicing variations. Analysis of the DNA sequences was performed using Sequencher v4.2 (Gene Codes, Ann Arbor, MI; www.genecodes.com).

Subcloning the PCR Product and Sequence Analysis

PCR products of potential mutant transcripts were purified as described above and were subcloned directly into a pCR 4-TOPO vector using the TA Cloning Kit for Sequencing (Invitrogen) following the manufacture's instruction. PCR was performed to identify bacterial colonies containing appropriate inserts. Plasmid DNA was purified using QIAfilter Plasmid Maxi Kit (Qiagen) and the insert was sequenced using either the universal M13-primers or the primers for PCR reactions.

Image Analysis and Statistical Analysis

The results of RT-PCR were quantified using the AlphaEase FC (Alpha Innotech, San Leandro, CA; www.alphainnotech.com) for the integrated density of each band. The Student's *t*-test was employed using SAS software 8.0 (SAS Institute, Cary, NC; www.sas.com). A value of $P<0.05$ was considered significant and results were presented as the mean ± standard deviation (SD).

RESULTS

BRCA1 and *BRCA2* Intronic Variants

As a result of the clinical genetic susceptibility testing of the 375 probands of breast/ovarian cancer-prone kindreds followed by FRAP, we identified several low-frequency sequence alterations in *BRCA1* and *BRCA2* that flanked the intron-exon boundaries (i.e., *BRCA1*, c.212+3A>G/IVS5+3A>G, c.301-2delA/IVS6-2delA, c.441+1G>A/IVS7+1G>A, c.593+8A>G/IVS9+8A>G, c.4986+6T>G/IVS16+6T>G, and c.4986-20A>G/IVS16-20A>G and *BRCA2*, c.516-19C>T/IVS6-19C>T, c.7976-4_7976_3delTT/IVS17-4delTT, c.8487+1G>A/IVS19+1G>A, c.8487+19A>G/IVS19+19A>G, c.8632-2A>G/IVS20-2A>G, and c.9256-18C>A/IVS24-18C>A). Supplementary Table S1A (available online at www.interscience.wiley.com/jpages/1059-7794/suppmat) lists the cancer history status of these families, and Supplementary Table S1B shows the number of times each alteration has been previously reported to the BIC database. Among these alterations, *BRCA1*-c.593+8A>G, *BRCA1*-c.441+1G>A, *BRCA2*-c.7976-4_7976_3delTT, and *BRCA2*-c.8487+19A>G are novel alterations and have not previously been reported. Five alterations, *BRCA1*-c.4986+6T>G, *BRCA1*-c.4986-20A>G, *BRCA2*-c.516-19C>T, *BRCA2*-c.9256-18C>A, and *BRCA2*-c.8632-2A>G, have been detected

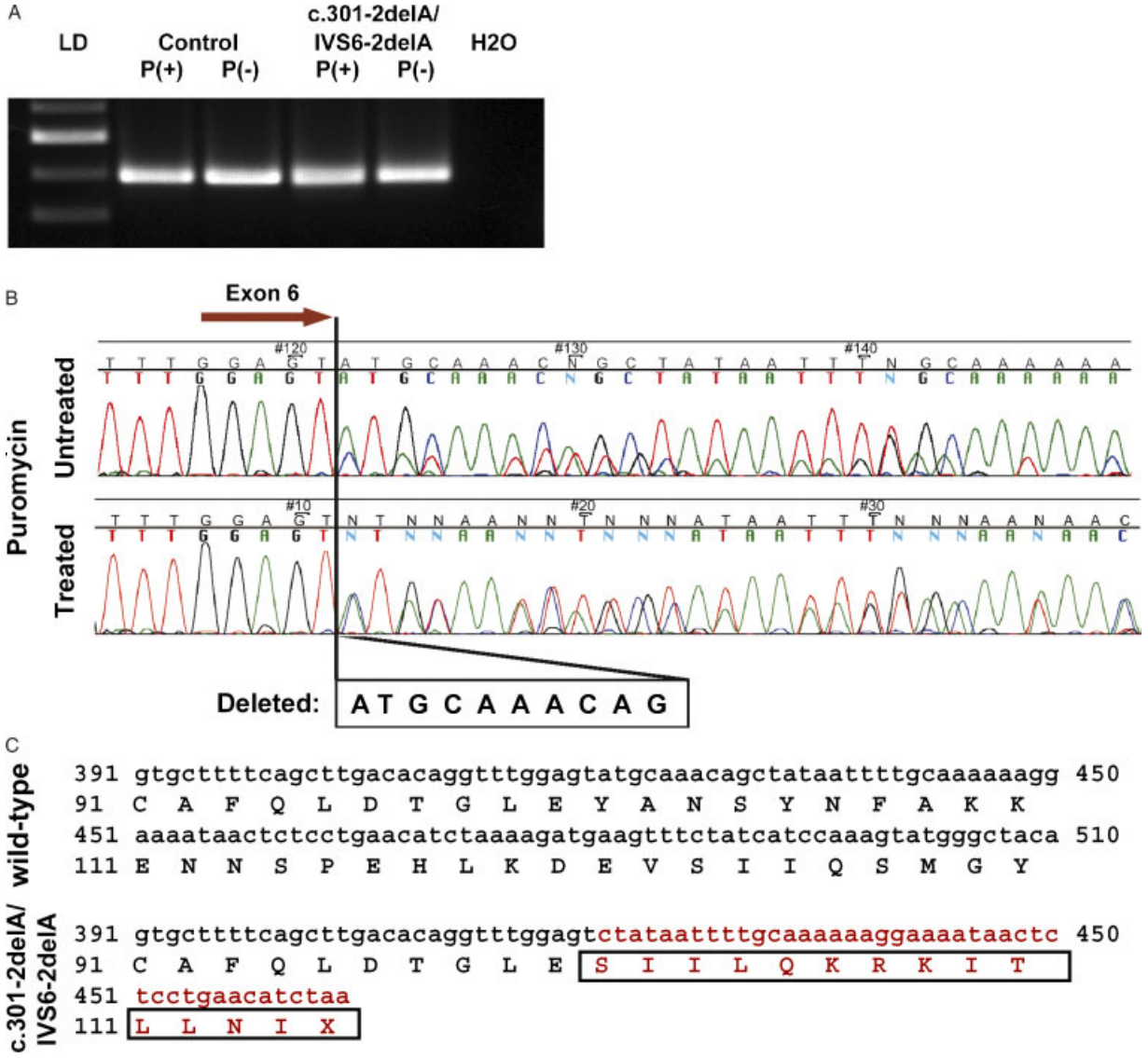

FIGURE 1. **Aberrant splicing associated with *BRCA1*-c.301-2delA/IVS6-2delA mutation. A:** RT-PCR analysis by agarose gel image. PCR samples were loaded from left to right as a DNA ladder, wild-type lymphoblastoid cells (Control) treated with puromycin (P(+)) and without puromycin (P(–)), mutation carrier lymphoblastoid cells treated with puromycin (P(+)) and without puromycin (P(–)), and negative control (H_2O). **B:** Sequencing analysis for sample from mutation carrier. *BRCA1*-c.301-2delA results in a 10-base frameshift deletion at the beginning of exon 7 (NM_007295.2:c.302_311del10). **C:** Protein sequencing analysis. The 10-bp frameshift deletion in the cDNA creates 14 new residues (101–114) and a predicted stop codon at residue 115 (NP_009226.1:p.Tyr101SerfsX15).

previously but their impact on mRNA splicing has not been investigated.

Aberrant Splicing Analysis by RT-PCR

To evaluate the consequence of each sequence variant on splicing, total RNA was isolated from cryopreserved lymphocyte pellets and/or LCLs. Primers used in the PCR reaction were designed to amplify the exon regions that are predicted to be affected by the splice-site mutations. In addition, to prevent potential degradation of unstable transcripts by nonsense-mediated mRNA decay, LCLs were treated with puromycin, a translational inhibitor, prior to RNA isolation. As shown in Supplementary Table S1B, five of the variants lead to expression of alternatively-spliced transcripts, while the other seven alterations have no apparent effects on mRNA splicing. Of these sequence variants, all but one involve the highly conserved splice donor (GT) or splice acceptor (AG) sequence flanking the intron-exon junction.

BRCA1-c.301-2delA has been reported to the BIC database, but its role in mRNA splicing has not been previously studied. In our studies, although the mutation carrier herself has no cancer, three family members have been diagnosed with breast cancer (Supplementary Fig. S1A). Analysis of cDNA products demonstrates that this intronic deletion activates another cryptic splice site, which

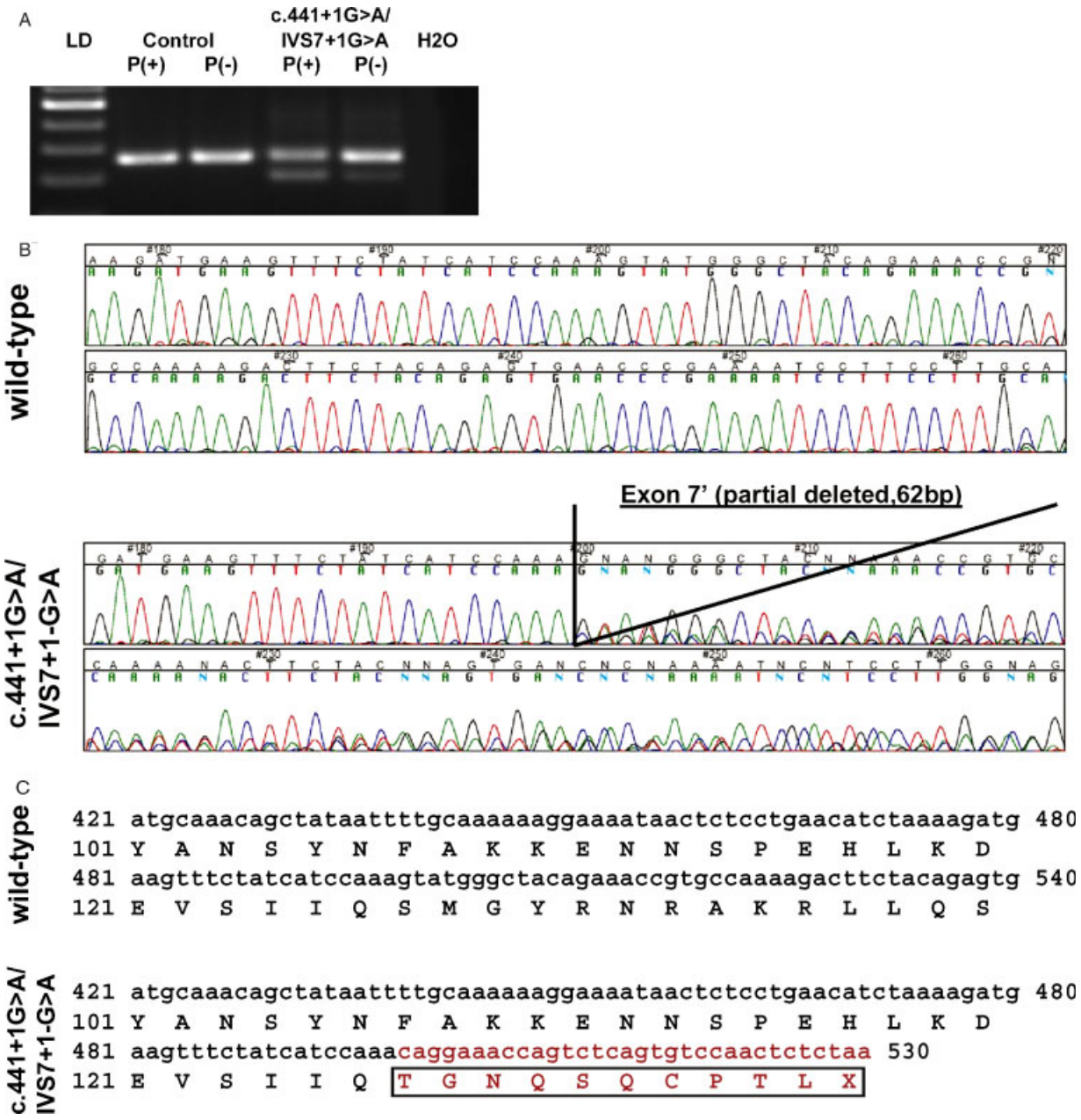

FIGURE 2. **Aberrant splicing associated with *BRCA1*-c.441+1G>A/IVS7+1G>A. A:** RT-PCR analysis by agarose gel image. PCR samples were loaded from left to right as a DNA ladder, wild-type lymphoblastoid cells (Control) treated with puromycin (P(+)) and without puromycin (P(–)), mutation carrier lymphoblastoid cells treated with puromycin (P(+)) and without puromycin (P(–)), and negative control (H_2O). **B:** Sequencing analysis for samples from wild-type and mutation carrier. The alteration *BRCA1*-c.441+1G>A. leads to a 62-bp deletion at the end of exon 7 (NM_007295.2:c.380_441del62). **C:** Protein sequencing analysis showed that this 62-bp frameshift deletion creates 10 new residues and a predicted stop codon at residue 137 (NP_009226.1:p.Ser127ThrfsX11).

results in a 10-bp frameshift deletion at the beginning of exon 7 (c.302_311del10). This frameshift deletion then results in the addition of 14 new residues (101–114) and a premature stop codon at residue 115 (p.Tyr101SerfsX15) (Fig. 1A–C). Because of only 10-bp differences between the wild-type and mutant bands, it is difficult to distinguish the wild-type and mutant bands. Therefore, the abundance of each was estimated by the peak strength in the sequence electropherograms, and the aberrant transcripts were more abundant in the puromycin treated vs. the untreated LCLs (Fig. 1B).

BRCA1-c.441+1G>A to our knowledge has not previously been reported. This alteration is detected in a proband diagnosed with breast cancer at 34 and 43 (Supplementary Fig. S1B).

TABLE 1. **Ratio of mRNA Expression of the Aberrant Spliced Alleles to the Wild-Type Alleles in LCLs Treated With or Without Puromycin**

	BRCA1			BRCA2	
	C.301-2delA[a]/ IVS6-2delA[b]	c.441+1G>A/ IVS7+1G>A	c.4986T>G/ IVS16+6T>G	c.8487+1G>A/ IVS19+1G>A	c.8632-2A>G/ IVS20-2A>G
Nontreated ±0.05[d]	–[c]	0.35±0.08	0.29±0.08	1.96±0.56	0.72±0.02/0.54
Treated ±0.15	–	0.69±0.06	0.59±0.10	1.97±0.56	0.96±0.13/1.00
P-value	–	0.03	0.03	NS	0.05/0.005

[a]RefSeqs (GenBank accession no. NM_007295.2 and NM_000059.1) are used for *BRCA1* and *BRCA2* numbering, respectively, and the A of the ATG translation initiation codon is +1, according to approved guidelines (www.hgvs.org/mutnomen).
[b]Traditional nomenclature used in BIC database, based on RefSeqs (GenBank accession no. U14680 and U43746) for *BRCA1* and *BRCA2* numbering, respectively.
[c]Because of 10-bp differences between the wild-type and mutant bands, the density was not able to be quantified.
[d]It represents the ratios of two aberrant spliced alleles to the wild-type alleles, NM_000059.1:c.[8632-1356_8632-1264ins; 8633_8675del] (upper band in Fig. 5A) and NM_000059.1:c.8633_8675del (lower band in Fig. 5A), respectively.

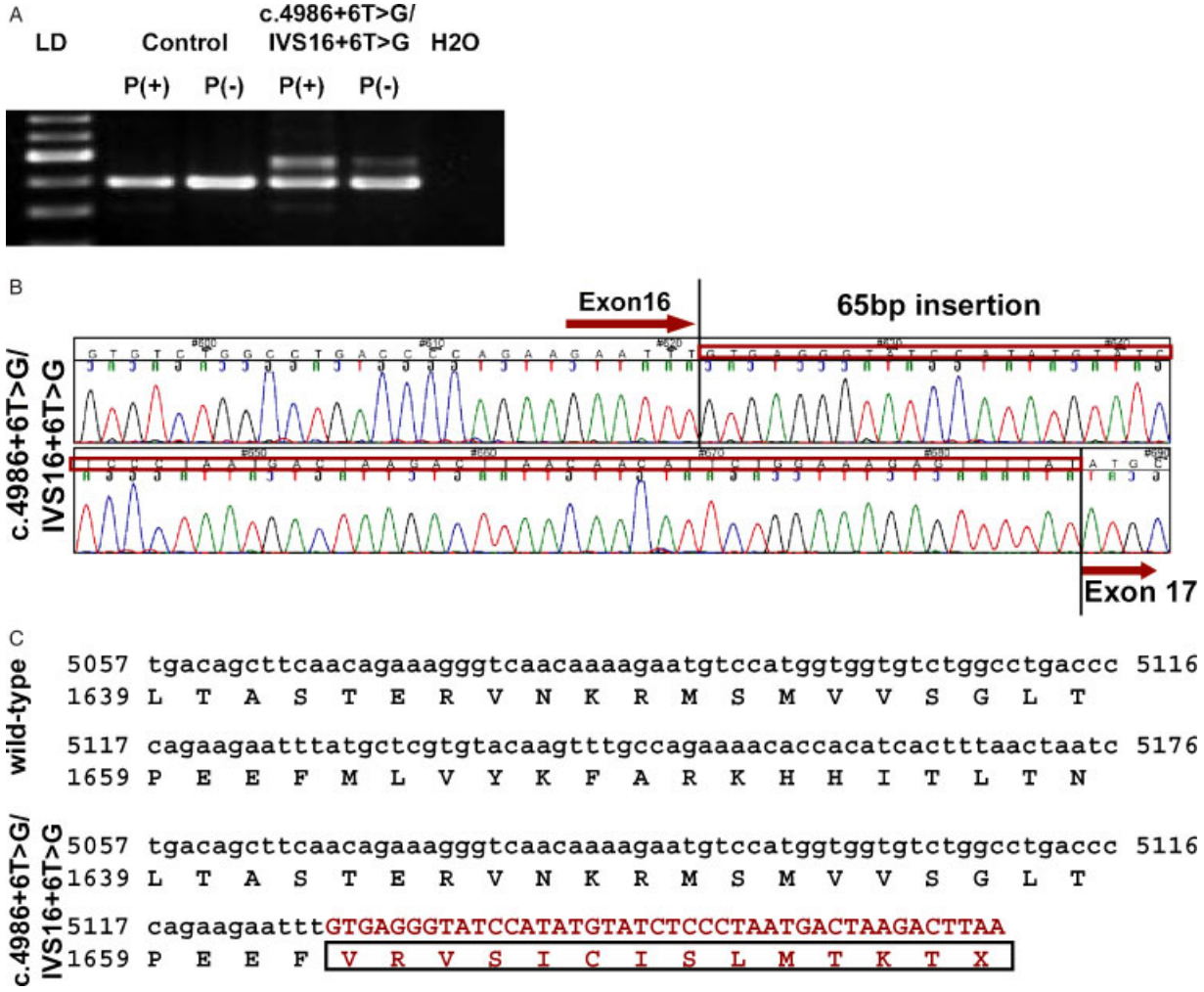

FIGURE 3. **Aberrant splicing associated with *BRCA1*-c.4986+6T>G/IVS16+6T>G. A:** RT-PCR analysis by agarose gel image. PCR samples were loaded from left to right as a DNA ladder, wild-type lymphoblastoid cells (Control) treated with puromycin (P(+)) and without puromycin (P(–)), mutation carrier lymphoblastoid cells treated with puromycin (P(+)) and without puromycin (P(–)), and negative control (H_2O). **B:** Sequencing analysis for samples from mutation carrier following subcloning into pCR4-TOPO. This alteration leads a 65-base insertion from intron 16 (NM_007295.2:c.4916+1_4916+65ins65). **C:** Protein sequencing analysis indicate this 65-bp insertion creates a predicted stop codon at residue 1676 (NP_009226.1:p.Met1663ValfsX14).

The inheritance of this mutation is unclear, since both the maternal and paternal side of the family report breast or ovarian cancer and no other samples were available from this kindred. Analysis of mRNA from the lymphocytes of this individual by RT-PCR showed that this alteration results in a 62-base deletion within exon 7 (c.380_441del62) (Fig. 2A–C). This frameshift is predicted to result in the addition of 10 amino acid residues and premature termination of translation at residue 137 (p.Ser127ThrfsX11) (Fig. 2C). The aberrant transcript is presented in both puromycin treated and untreated LCLs. However, the mRNA expression ratios of mutant alleles to wild-type alleles are approximately 0.4:1 and 0.7:1 in puromycin untreated and treated LCLs, respectively ($P < 0.03$) (Fig. 2A; Table 1).

BRCA1-c.4986+6T>G has been reported previously, but has not been functionally characterized. The proband was diagnosed with ovarian cancer at age of 62, and reported a strong family history of breast and ovarian cancer (Supplementary Fig. S1C). We performed RT-PCR to amplify the region from exon 17 to 22 using RNA samples isolated from peripheral lymphocytes of the alteration carrier and cancer-free controls and demonstrated that this alteration leads to an aberrant *BRCA1* transcript, which incorporates 65 bases of intron 16 sequence, immediately following exon 16 (c.4916+1_4916+65ins65) (Fig. 3A–C). Sequence analysis indicates the mutation results in the activation of a cryptic splice site at g.38,476,405 (GenBank accession no. NC_000017.9). This 65-base insertion is predicted to create a stop codon at residue 1676, plus 13 additional residues encoded by the inserted sequence (p.Met1663ValfsX14). The mRNA expression ratios of mutant alleles to wild-type alleles are approximately 0.3:1 and 0.6:1 in puromycin untreated and treated LCLs, respectively ($P < 0.03$) (Fig. 3A; Table 1).

BRCA2-c.8487+1G>A has been previously reported to the BIC database. For this family, the proband was diagnosed with invasive breast cancer at age 41, and she reported two paternal aunts and a paternal grandmother with breast cancer (Supplementary Fig. S1D). RT-PCR analysis of RNA from the proband's peripheral lymphocytes found that exon 19 (156 bp) was deleted (c.8332_8487del156), resulting in an in-frame deletion of 52 amino acids (p.Ile2778_Gln2829del52) (Fig. 4A and B). Interestingly, the level of the mutant *BRCA2* transcript appears to be much more abundant than the wild-type transcript. The mRNA expression ratios of mutant alleles to wild-type alleles are almost identical (1.96:1 vs. 1.97:1) in puromycin untreated and treated LCLs, respectively (Fig. 4A; Table 1).

BRCA2-c.8632-2A>G has been reported one time previously to BIC. The proband in this family was diagnosed with ovarian cancer at age 39 and reported two sisters with breast cancer at 34 and 35 years of age (Supplementary Fig. S1E). As before, we used RT-PCR to amplify the region from exon 19 to 22. We found that the *BRCA2*-c.8632-2A>G mutation leads to two splicing variants, a 43-bp deletion at the beginning of exon 21 (c.8633_8675del43) or a 93-bp insertion from intron 20 in

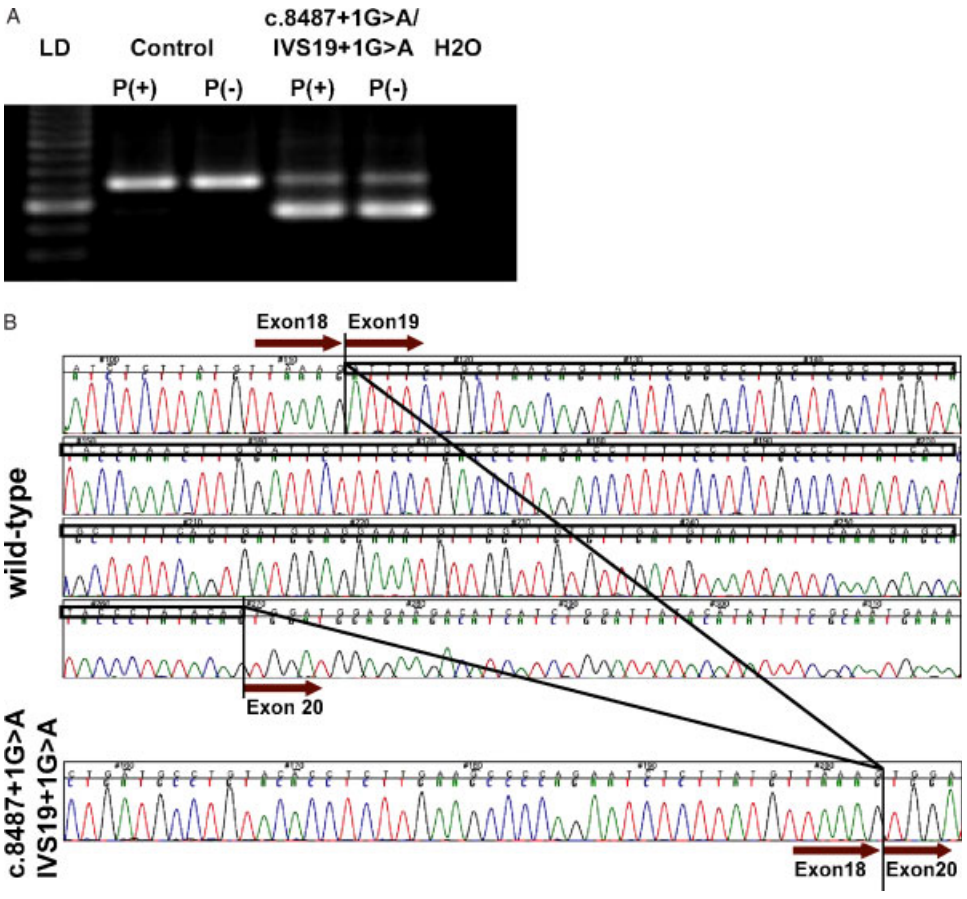

FIGURE 4. **Aberrant splicing associated with *BRCA2*-c.8487+1G>A/IVS19+1G>A. A: RT-PCR analysis by agarose gel image. PCR samples were loaded from left to right as a DNA ladder, wild-type lymphoblastoid cells (Control) treated with puromycin (P(+)) and without puromycin (P(–)), mutation carrier lymphoblastoid cells treated with puromycin (P(+)) and without puromycin (P(–)), and negative control (H_2O). B: Sequencing analysis for samples from wild-type and mutation carrier by subcloning. *BRCA2*-c.8487+1G>A allele skips exon 19 (NM_ 000059.1:c.8332_8487del156).**

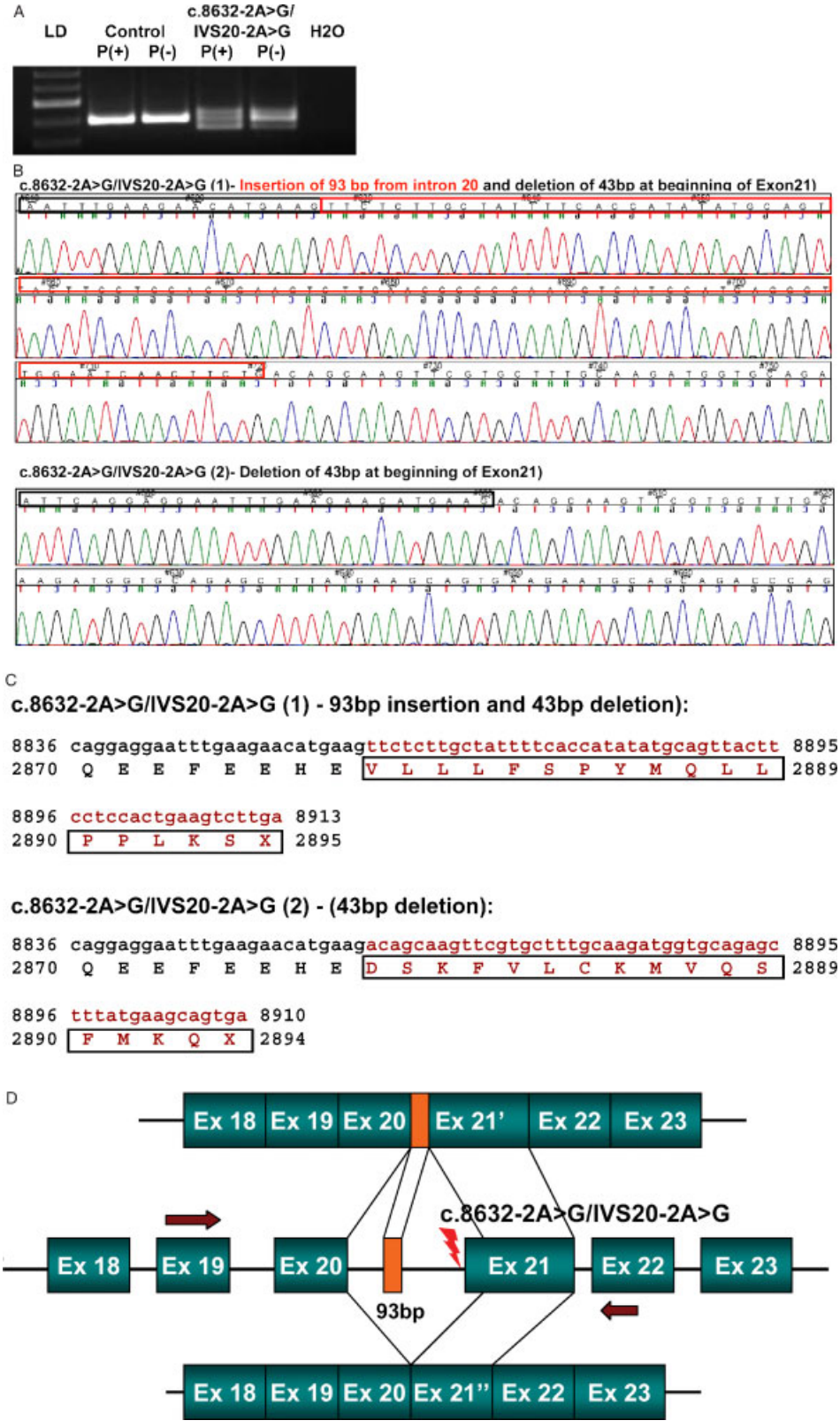

FIGURE 5. **Aberrant splicing associated with *BRCA2*-c.8632–2A>G/IVS20–2A>G. A:** RT-PCR analysis by agarose gel image. PCR samples were loaded from left to right as a DNA ladder, wild-type lymphoblastoid cells (Control) treated with puromycin (P(+)) and without puromycin (P(–)), mutation carrier lymphoblastoid cells treated with puromycin (P(+)) and without puromycin (P(–)), and negative control (H_2O). **B:** Sequencing analysis for samples from mutation carrier by subcloning. The alteration of *BRCA2*-c.8632–2A>G leads to either the 93-bp insertion from intron 20 and the 43-bp deletion of exon 21 (NM_ 000059.1:c.[8632-1356_8632-1264ins93; 8633_8675del43]), or the 43-bp deletion at the beginning of exon 21 (NM_ 000059.1:c.8633_8675del43). **C:** Protein sequencing analysis indicates that *BRCA2*-c.8632-2A>G causes two distinct BRCA2 truncated proteins, NP_000050.1:p.Glu2878ValfsX18 and p.Glu2878AspfsX17. **D:** Schematics for aberrant splicing caused by mutation *BRCA2*-c.8632–2A>G.

combination with the 43-bp deletion of exon 21 (c.[8632-1356_8632-1264ins93; 8633_8675del43], GenBank Accession No: NC_000013.9 and NM_000059.1), which are both predicted to lead to truncated proteins, p.Glu2878ValfsX18 and p.Glu2878AspfsX17, respectively (Fig. 5A–D). The mRNA expression ratios of mutant alleles to wild-type alleles (c.[8632-1356_8632-1264ins93; 8633_8675del43], i.e., upper band) is approximately 0.7:1 and 1:1 in puromycin untreated and treated LCLs, respectively ($P < 0.05$). The mRNA expression ratio of mutant alleles to wild-type alleles (c.8633_8675del43, i.e., lower band) is 0.5:1 and 1:1 in puromycin untreated and treated LCLs, respectively ($P < 0.005$) (Fig. 5A; Table 1).

DISCUSSION

Since *BRCA1* and *BRCA2* were cloned and characterized in the mid-1990s [Frank et al., 2002; Miki et al., 1994; Wooster et al., 1995], hundreds of different sequence variants in each of these genes have been detected, including a large number of the intronic variants. We characterized the potential pathological significance of 6 *BRCA1* and 6 *BRCA2* intronic alterations at the mRNA level. All of the sequence alterations are located within or near consensus splicing sites. We detected aberrantly spliced transcripts for three of the *BRCA1* variants (c.301-2delA, c.441+1G>A/IVS7+1G>A, and c.4986+6T>G) and two of the *BRCA2* variants (c.8487+1G>A and c.8632-2A>G). The other *BRCA1* and *BRCA2* variants show no effects on RNA splicing.

In our efforts to classify *BRCA1* and *BRCA2* variant transcripts, we found that treatment of LCLs with puromycin helped facilitate the analysis of the mutant transcripts. Because of potential degradation of unstable transcripts by mRNA NMD [Losson and Lacroute, 1979; Perrin-Vidoz et al., 2002], the aberrant transcripts identified in the LCLs treated with puromycin may be considerably less abundant or even absent in the nontreated LCLs and peripheral lymphocytes. Except for the aberrant transcripts associated with the *BRCA2*-c.8487+1G>A, the other aberrant transcripts identified in our study are weakly expressed (*BRCA1*-c.301-2delA, *BRCA1*-c.441+1G>A, *BRCA1*-c.4986+6T>G, and *BRCA2*-c.8632-2A>G) in comparison to the wild-type transcripts (Table 1). The common characteristics shared by these four alterations are that they all result in a frameshift in the translational reading frame and a PTC. In comparison, *BRCA2*-c.8487+1G>A is predicted to result in a 52-amino acid in-frame deletion that does not cause a PTC (Fig. 4A and B). Furthermore, we examine the NMD rule—"PTCs before ~50–55-bp of the end of the penultimate exon initiate NMD"—and all the PTCs containing alleles in our studies comply with this 50–55 nucleotide rule (data not shown). These results suggest that the mutated *BRCA1* and *BRCA2* transcripts that create premature stop codons are less stable. Therefore, *BRCA1* and *BRCA2* aberrant transcripts eliminated by NMD could result in downregulating the overall level of both mRNA and protein of BRCA1 and BRCA2. Loss of BRCA1 expression has been reported to be related to the pathogenesis of breast cancer [Thompson et al., 1995; Wilson et al., 1999; Zheng et al., 2000; Wei et al., 2005].

In conclusion, our study has evaluated several *BRCA1* and *BRCA2* variants located within or near intron-exon boundaries. Seven of them have no apparent impact on the mRNA splicing process, four cause frameshift mutation, and one resulted in an in-frame deletion. These studies help to confirm whether alterations in *BRCA1* and *BRCA2* are likely to be benign polymorphisms or pathogenic mutations. As our study is fundamentally based on the analysis of mRNA transcripts, it will be helpful to carry out further research at the protein level since many of the mutant alleles could be reduced in expression relative to wild-type indicating that loss of BRCA1 or BRCA2 protein may contribute to the pathogenesis of breast and/or ovarian cancer rather than expression of a defective protein.

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